IMPERIAL AGRICULTURAL
RESEARCH INSTITUTE, NEW DELHI.

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COLIFORM INTERMEDIATES IN HUMAN FECES

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Received for publication November 30, 1937

Although foundation for the recognition of coliform intermediates was laid by Brown (1921), he did not utilize his findings for their demonstration. Brown was interested in mediums containing citrated blood and attempted to determine the effect citrate might have on the growth of organisms likely to be encountered in blood culture work. Koser (1923, 1924a, b, c, d, 1926) applied to coliform bacteria (Breed and Norton, 1937) tests involving utilization of organic acids and their salts. He found that *Bacterium coli* from feces could not utilize citrate as a sole carbon source in contrast to *Bacterium aerogenes*. Koser found, particularly in soil and water, organisms classed as coli by the criteria then used but which utilized citrate-carbon and, in this respect, resembled *B. aerogenes*. In 1924 Koser applied the term "intermediate" to these forms.

Recognition of such intermediate forms should make possible a better theoretical understanding of the entire coliform group, than which few in bacteriology are more complex or confused. Any fundamental advance, such as the intermediate concept, should enable sanitary science to define more accurately such terms as "pollution" and "potability" and to answer numerous practical questions posed in this field in recent years.

Parr (1936a, b, 1937) defined intermediates as coliform organisms "which have one or more coli characters and one or more of those attributed to aerogenes, and some 'intermediates,' including the typical fecal form, produce hydrogen sulphide." This definition permits the inclusion as intermediates of organisms which are Voges-Proskauer positive, a feature we feel essential and justified by our data.

The three characteristics, defining coli and aerogenes, most emphasized in recent papers are the methyl-red and Voges-Proskauer reactions and the utilization of citrate. To these it is felt indol production should be added. Formerly, following Houston, a water was considered polluted if it gave a "lactose +, indol +" reaction and the "Flaginac" mnemonic of English sanitarians, defining the colon bacillus, analyzes into "FL," fluorescence in neutral-red broth; "AG," acid and gas in lactose broth; "IN," indol from tryptophane; and "AC," acidity with coagulation in milk.

Parr and Caldwell (1933a, b) did not include the Voges-Proskauer reaction, under the impression that this reaction shows perfect correlation with the methyl-red reaction (Levine, 1916) and for the sake of uniformity the bored latrine studies by Caldwell and Parr (1937) utilize the same classification. It is now known that many coliform organisms do not exhibit perfect correlation, that is, they are not necessarily methyl-red positive when Voges-Proskauer negative and vice versa. Hence, in the present project four tests, i.e., indol production, methyl-red and Voges-Proskauer reactions, and citrate utilization were used as fundamental differentials in the classification of coliform organisms into coli, intermediates and aerogenes.

To fix and facilitate expression of results we devised the mnemonic "Imvic," (Parr, 1936b). Thus *Bacterium coli* (*Escherichia coli*) is + + − −, which means that it is indol positive (I), methyl-red positive (M), Voges-Proskauer negative (V), and citrate negative (C). *Bacterium aerogenes* (*Aerobacter aerogenes*) is − − + +, which means that it is indol negative, methyl-red negative, Voges-Proskauer positive and citrate positive. Table 1, a compilation of the tests used by some of the workers in this field in the order in which they list the tests used, is evidence for the need of some such standardization and justification for the selection of the members of the Imvic quartet of tests.

The 16 types, which combinations of the four characters determine, are listed in table 2. In this table the types encountered by recent investigators are indicated.

TABLE 1

Summary of differential tests used in the study of coliform intermediates

Koser (1924)	M.R.	V.P.	Uric A.	Citrate
Kline (1930)	M.R.	V.P.	Uric A.	Citrate
Ruchhoft et al. (1931)	Indol	M.R.	V.P.	Citrate
Gray (1932)	M.R.	V.P.	Citrate	
Skinner and Brudnoy (1932)	Cello. V.P.	Citrate M.R.	Indol	Sucrose
Parr and Caldwell (1933)	M.R.	Indol	Citrate	
Bardsley (1934)	M.R. Citrate	V.P.	Indol	Uric A.
Bigger (1934)	Indol	M.R.	V.P.	Citrate
Tittsler and Sandholzer (1935)	Cello.	Citrate	H_2S	A-M-D-G
Wilson et al. (1935)	M.R. Eijkman	V.P. Gelatin	Citrate	Indol
Raghavachari and Iyer (1935)	M.R.	V.P.	Indol	Citrate
Kline (1935)	M.R. Eijkman	V.P.	Citrate	Indol
Griffiths and Fuller (1936)	V.P. Indol	M.R.	Citrate	Uric A.
Parr (1936)	Indol	M.R.	V.P.	Citrate
Bartram and Black (1937)	M.R.	V.P.	Citrate	Indol
Hook and Hitchener (1937)	M.R. Cello.	V.P.	Indol	Citrate
Carpenter and Fulton (1937)	Citrate	M.R.	V.P.	

Times the test appears in this tabulation:

Indol production	12
Methyl-red reaction	16
Voges-Proskauer reaction	15
Citrate utilization	17
Uric acid utilization	4
Cellobiose fermentation	3
Eijkman test	2
Hydrogen sulphide production	1
Gelatin liquefaction	1
Sucrose fermentation	1
Alpha-methyl-d-glucoside fermentation	1

This report is primarily concerned with coliform intermediates derived from three types of fecal material, i.e., specimens of fresh feces and certain of these specimens stored at ice box and at body temperatures. Throughout, isolation of cultures has been by direct plating. True, for each fresh specimen enrichment tubes

TABLE 2

Coliform types and their occurrence

	1	2	3	4	5	6	7	8	9
Coli									
++−−	*	+	+	+	+	+	+	+	+
−+−−			+	+	+	+	+	+	+
+−−−									
Intermediates									
+++−					+				+
++−+			+	+	+	+	+	+	+
−+−+		+	+	+	+	+	+	+	+
+−+−									
−−−−								+	
+−−+			+	+				+	
−++−			+				+		+
++++				+			+		+
+−++		+	+	+		+		+	+
−+++			+	+			+		+
Aerogenes									
−−−+			+	+			+		
−−+−			+			+	+	+	+
−−++		+	+	+	+	+	+	+	+

The formulae express in order the indol production, methyl-red reaction, Voges-Proskauer reaction and citrate utilization, i.e., the "Imvic reaction."

* 1. Koser reported five types, but did not utilize indol. Types cannot be exactly placed.

2. Minkewitsch.
3. Kline.
4. Ruchhoft et al. Recognized but four types, ++−−, −+−−, −+−+, and −−++ as fecal. Others held to be extraneous to feces or mixtures.
5. Skinner and Brudnoy.
6. Bardsley.
7. Bigger.
8. Wilson et al.
9. Parr.

of lactose broth were prepared, but they were not utilized unless direct plating failed to reveal coliform organisms. That this may happen has been shown by Parr (in press) and by Carpenter and Fulton (1937) who record occasional fecal specimens which yield no coliform bacteria. It is felt that direct plating is the most accurate method for determining the actual flora present. The various coliform bacteria have different metabolic demands and responses. It is improbable that any enrichment method yields an entirely accurate picture of the material enriched.

The colonies chosen for study have been purified by serial inoculation into plain broth and plating on Endo's agar. Ruchhoft and co-workers (1931) rendered a valuable service in emphasizing the confusion which mixed cultures introduce into the coliform field. Due cognizance has been taken of this point.

TABLE 3

Strains repeatedly replated to check purity

TYPE	STRAINS TESTED	CHANGES
−+++	3	0
++−+	5	0
++++	6	0
+−++	6	2*
−−+−	2	0
++−−	1	0
−+−+	2	0

* At the close of the experiment the two strains of +−++ which had changed were recovered as −−++. When mixtures are plated one usually recovers the component strains. For these none was encountered.

Strains isolated which did not correspond with the types sanctioned by the Ruchhoft report were repurified and retested. In addition, as a check on the Ruchhoft point of view, which is that only four fecal coliform types exist, i.e., −+−−, ++−−, −−++, and −+−+, a number of strains were further subjected to detailed purification involving 28 serial transplants on various mediums including seven platings and pickings. In only two instances did the reactions change and in these cases we are not convinced that the cultures in question were mixtures. See table 3.

As Parr and Caldwell (1933b) and d'Herelle and Rakieten (1934) have suggested, the possibility of biochemical variation, in freshly isolated strains not yet acclimatized to laboratory mediums, cannot be overlooked. Among the coliform bacteria characters are from time to time lost or those in abeyance are regained. It is in part this property of the group that gives rise to the multiplicity of forms responsible for confusing results in the attempt to apply a rigid and detailed botanical type of taxonomy and makes it desirable to emphasize the "lumper" point of view rather than the "splitter" type of classification (Skinner and Brudnoy, 1932). The occasional appearance of a variant reaction may, in our opinion, be a tribute to an investigator's close touch with his strains rather than, a priori, evidence of his carelessness. Wilson and co-workers (1935) stated that most cultures positive to both the methyl-red and Voges-Proskauer reactions are mixtures. Our results (table 3 and table 4) do not confirm this view.

Purified cultures were tested according to the Imvic complex and in addition for their dissimilation of glucose, lactose, sucrose, dulcitol, inositol, salicin, cellobiose and alpha-methyl-d-glycoside, for the production of hydrogen sulphide, liquefaction of gelatin and action on milk. Gram and capsule stains and motility tests were made as indicated. In addition, a considerable number of strains were examined for their growth in boric-acid lactose broth, in sodium malonate broth, and in Jordan and Harmon's tartrate agar; for their reduction of methylene blue and for their fermentation of propylene glycol, adonitol, inulin, raffinose, mannitol, arabinose, rhamnose, maltose and xylose. Endo's agar was used for plating, Simmon's citrated agar for determination of citrate utilization and fermentation broths were prepared from Difco phenol-red broth base to which the requisite carbohydrate, glucoside or alcohol was added prior to autoclave sterilization. Levine's iron citrate medium was used for hydrogen sulphide determinations. The data on coliform intermediates from human feces are presented in table 4.

Tribute to the heterogeneity of the coliform intermediates has been paid by Werkman and Gillen (1932), Tittsler and Sandholzer (1935), Parr (1936a) and by Carpenter and Fulton (1937). In each case the reference is to intermediates of the − + − + type which our data clearly show is the most important type.

In our collection of intermediates of this type we have 29 varieties. Of these, 21 are hydrogen-sulphide positive, four liquefy gelatin, one fails to ferment cellobiose, 14 fail to ferment alpha-methyl-d-glucoside and, when analyzed by their fermentations of sucrose and dulcitol into MacConkey types, six are "acidi-lactici," six are "communis," eight are "communior," and nine are "aerogenes." This collection of −+−+ types includes strains from other than fecal sources, such as "infected pumps," milk, marine food, eggs, soil, water and animal pathology.

TABLE 4

Fecal coliform intermediates

		FRESH FECES	ICE BOX STORAGE	37°C. STORAGE	TOTAL
Specimens		235	68	38	
Platings		235	351	183	
Strains studied		1987	1690	905	4582
Intermediates		153	473	139	765
Per cent		7.7	27.9	15.3	16.6
Types	+++−	1	0	0	1
	++−+	7	81	23	111
	−+−+	110	135	59	304
	−++−	0	6	0	6
	++++	7	122	9	138
	+−++	28	50	39	117
	−+++	0	79	9	88
Totals		153	473	139	765

Order of importance:

(1)	−+−+	−+−+	−+−+	−+−+
(2)	+−++	++++	+−++	++++
(3)	++−+ ++++	++−+	++−+	+−++
(4)		−+++	++++ −+++	++−+
(5)		+−++		−+++

By contrast the intermediate types −+++, +−++, and ++++ are quite homogeneous whereas the type ++−+ though less heterogeneous than −+−+ is nevertheless much more so than −+++, +−++, or ++++. None of these intermediate types belongs to but one MacConkey group. All −+++, +−++ and ++++ strains and all but one ++−+ strain we have encountered ferment sucrose. The split which gives rise to more than one MacConkey group is found in the dulcitol fermentation. All these types, then, are found as both "communior" and "aerogenes" and in addition we have encountered a "communis" type ++−+. The four intermediate types in question ferment cellobiose and alpha-methyl-d-glucoside and do not liquefy gelatin or produce hydrogen sulphide.

TABLE 5

Tabulation of tests designed to reveal coliform relationships

TYPES	M.B.-REDUCTION			PROPYLENE GLYCOL			SODIUM MALONATE			ADONITOL			BORIC ACID		
	+	−	Total	+	−	Total	+	−	Total	+	−	Total	+	−	Total
++−−	10	1	11	2	78	80	0	125	125	15	64	79	204	0	204
−+−−	0	0	0	0	13	13	0	19	19	7	4	11	5	0	5
++−+	5	6	11	4	6	10	6	4	10	8	28	36	16	0	16
−+−+	34	28	62	46	14	60	11	63	74	2	164	166	40	0	40
++++	3	11	14	11	0	11	14	2	16	36	0	36	5	0	5
+−++	6	0	6	9	0	9	12	0	12	14	0	14	1	0	1
−+++	14	2	16	8	2	10	19	2	21	43	0	43	3	0	3
−−+−	4	2	6	14	0	14	0	14	14	0	16	16	0	0	0
−−++	5	0	5	46	11	57	64	2	66	5	16	21	48	15	63
−−−+	0	0	0	2	0	2	2	0	2	1	0	1	0	0	0
Totals....	81	50	131	142	124	266	128	231	359	131	292	423	322	15	337

(Exceptions in the ++−+ type). All −+++, +−++, and ++++ ferment inositol and adonitol whereas the ++−+ does not ferment inositol and most strains fail to ferment adonitol. It is interesting to note that the heterogeneous −+−+ type exhibits more homogeneity with respect to adonitol dissimilation than to any other test of differential value we have encountered, all but two of 166 strains failing to ferment that alcohol. The +−++ type is called aerogenes by a few workers. Table 5 gives the data for adonitol fermentation for some of the strains studied and includes other fragments of data which may interest students of the intermediates.

An important question is that of classification of the coliform intermediates. On this point Koser did not commit himself. Minkewitsch (1930) would recognize five species of coliform organisms as follows:

Coli sub-group:
  Type I. *B. coli-communis* Escherich. Including all of its varieties and even the lactose-defective races of paracoli which are connected with typical coli by the intermediate form *B. coli-mutabile.*
  Type II. *B. coli-citrovorum* Koser. Includes only the – + – + type of intermediate.
  Type III. *B. coli-anaerogenes* Lembke. Acid produced, but no gas.

Aerogenes sub-group:
  Type IV. *B. aerogenes* Escherich.
  Type V. *B. cloacae* Jordan.

Minkewitsch's terminology requires, of course, a change in genus from *Bacillus* to *Bacterium* and we feel his intermediate species is too narrowly conceived. His concept of the inclusion of paracoli and mutating coli with typical coli seems an excellent idea. We question whether his restriction of the intermediates, the anaerogenous coli and the cloacae to a habitat in cold-blooded animals can be justified. Furthermore it is hard to justify the establishment of a coli-anaerogenes species when paracoli are included with coli. It is to be regretted that in the coliform field no application can be made of animal pathology tests and that immunological procedures have as yet found but little utilization.

Werkman and Gillen (1932) have sought to erect a genus *Citrobacter* for organisms of the coliform group producing trimethylene glycol. They regard citrate utilization, the most widely used test in dealing with intermediates, as opposed to allocation with the citrate-negative colon bacilli.

Tittsler and Sandholzer (1935) divide the coliform bacteria into the genera *Escherichia* and *Aerobacter* on the basis of the

Voges-Proskauer reaction. As they conceive them the intermediates would then be classified as *Escherichia.* Although we cannot agree with their definition of intermediates their solution of the problem of classification could be accepted, some intermediates going to *Escherichia* and some to *Aerobacter.* More fundamental, as we see it, are the objections to breaking down the genus *Bacterium*; to including citrate-positive organisms with the coli; and to the emphasis placed on the Voges-Proskauer reaction, which is based on a quantitative rather than a qualitative differentiation. Carpenter and Fulton (1937) favor the inclusion of coliform intermediates in existing genera. They hold that coli and aerogenes are distinctly different and they believe the utilization of citrate not fundamental enough to prevent classing intermediates in the same genus with coli. They suggest a classification based primarily on the Voges-Proskauer reaction, secondarily on citrate.

Our experience compels us to regard citrate utilization as a sounder basis for classification than the Voges-Proskauer reaction. Koser (1924c) made a careful study of the stability of the utilization of citrate test. He found that under a wide variety of conditions the ability to utilize citrate is not readily lost when present or acquired when not present. With this conclusion we are in substantial agreement.[1]

It is further felt that cloacal forms of aerogenes differ sufficiently from the typical aerogenes so that any consideration of separate genera should consider a split in that direction. The gelatin-liquefying, motile, unencapsulated, glycerol-negative, propylene-glycol-negative, acetyl-methyl-carbinol-negative (as regards fermentation), chinic-acid (Butcher, 1926) negative cloacae demand taxonomic contrast to the aerogenes type with these reactions reversed. Jordan (11th Edition, 1935) felt that the splitting of the genus *Bacterium* into *Escherichia* and *Aerobacter* was not warranted. To this point of view we also subscribe.

Levine (1918) emphasized the desirability for restricting species numbers through a rigid application of methods of correlation. Unless points emerge distinctly advantageous for dairy, food and soil science, for sanitation and for medicine by the erection of new genera and species their construction seems unwise. This point is particularly pertinent for bacteriology, in which field botanical and zoological methods of classification only go so far, beyond which the microbes are lifted from their natural habitat and subjected to a wide variety of tests, selection of which has to be made with the greatest of care in order to elicit data of real value and to avoid undue complexity and confusion.

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[1] Rare exceptions which we have encountered are under study and will be the subject of a later report.

Among bacteria life spans are so short that many generations pass before the observer in a few days and without the averaging, leveling influence of sexual conjugation. In a group like the coliform bacteria where contributions by animal pathology and immunological methods are largely lacking and where opportunity for bacteriophage activity is so great it seems desirable to define the organisms encountered in simple and inclusive terms. This we feel could be done with four species of the genus *Bacterium* which would include coli, intermediates, aerogenes and cloacae and their varieties.

The coliform intermediates cannot be restricted to non-human sources as Minkewitsch (1930) has suggested. In a study of the coliform flora of 446 fecal specimens Carpenter and Fulton (1937) found one in which the only organism present was an intermediate type; 14 specimens where only intermediates and aerogenes were present; 16 yielding only intermediates and coli; and 31 which contained intermediates, acrogenes and coli. Thus they found intermediates in 62 specimens of 466 studied (13.3 per cent). Parr (In press) working on the general problem of the entire coliform flora and with a wider definition of intermediates than Carpenter and Fulton used found that 21.6 per cent of 235 fecal specimens contained intermediates. Of the 100 persons submitting these 235 specimens, 31 had a flora at one time or another containing intermediates. Parr found one specimen containing only intermediates, five containing aerogenes and intermediates, 25 containing intermediates and coli, and 19 specimens contained all three sections of the coliform group.

Parr (In press) has observed that there is considerable variation in the coliform flora of the same individual from day to day. Occasionally no coliform organisms at all may be recovered. Following such a period of time, which may include several days, coliform organisms again appear. It is at such "flora crises" as these that one is apt to encounter intermediates in very large numbers. They seem to be a stage in the re-establishment or balancing of the coliform flora. At other times they are not present at all or only in small numbers. Parr feels there is evidence for believing that the human colon is not the ideal habitat for coliform intermediates. However, since they are present in from one in eight to one in four of all fecal specimens and at times in very large numbers they cannot be stripped of significance in sanitation as indicators of pollution with alvine discharges.

It is unfortunate that our knowledge of coliform intermediates does not simplify the problems of sanitary science. That such problems are somewhat clarified is true, for added evidence is given in favor of the point of view that all coliform organisms may be suspected as of human fecal origin. The occurrence of all types of coliform bacteria in the bowel is further evidence of their close and intergrading relationship. From the practical standpoint evidence points to the necessity for strict emphasis on the highest standards of personal and public hygiene as the only safeguard against the spread of enteric bacteria.

CONCLUSION

A study has been made of 765 strains of coliform intermediates derived from fresh and stored feces and of additional type strains from other sources. As a working basis for the study of coliform organisms four tests have been utilized, i.e., indol production, methyl-red reaction, Voges-Proskauer reaction, and citrate utilization. All organisms are theoretically to be considered as intermediates which occupy a position between coli and aerogenes possessing one or more characteristics of coli and one or more of aerogenes. In practice we have restricted the intermediates to those so classified by the four reactions used.

The complexity of the entire coliform group is recognized. It is believed unwise to dignify the many differences between forms observed with taxonomic recognition. Classification for the entire group should be simple and might well be comprehended in four species of the genus *Bacterium* to include coli, intermediates, aerogenes and cloacae.

Whether coliform intermediates are essentially of fecal or nonfecal origin has not been determined. But it is certain that they do occur in stool specimens to such an extent that they must be considered for sanitary purposes as indicators of fecal pollution.

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THE EFFECTS OF SUBLETHAL DOSES OF MONOCHROMATIC ULTRAVIOLET RADIATION ON THE GROWTH PROPERTIES OF BACTERIA

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Received for publication November 30, 1937

INTRODUCTION

In earlier publications (Duggar and Hollaender, 1934; Hollaender and Claus, 1936; Hollaender and Duggar, 1936) attention has been directed to certain effects of monochromatic ultraviolet radiation on bacteria and to the technique employed in this laboratory for such studies. Thus far there have been considered almost exclusively the bactericidal effects; the technique of liquid suspension cultures, including the preparation and conduct of exposure and of control cultures; and the determination of survival ratios as manifested in colony development.

The purpose of the present paper is to present certain detailed data on some of the non-lethal effects of ultraviolet irradiation and to attempt to analyze these. Since the procedures used are outlined in our previous work, only certain important modifications are mentioned here. The data given represent only a very small but typical fraction of the experimental results. Two effects were consistently apparent in all experiments in which the specified conditioning factors were maintained, and these two effects are made apparent by a comparison of growth curves of irradiated and control organisms.

MATERIALS AND METHODS

The larger part of the work was done with a strain of *Escherichia coli* furnished by Dr. P. Levine and used in this laboratory for several years. The main features of the results obtained were duplicated with *Serratia marcescens.* The organisms were grown on nutrient (potato-glucose) agar slants, transferred daily (except as older cultures were desired), and used after 19 to 39 hours. For any possible advantage that might be derived therefrom, the *E. coli* culture was transferred, every four weeks, to a liquid beef-broth medium, and then back again, as usual, to potato-glucose slants. For exposure the bacteria were either washed off the agar slants with a physiological salt solution,[2] shaken thoroughly, filtered several times without pressure through absorbent cotton, and used directly for exposure; or, they were washed twice, centrifuged, resuspended, shaken, and filtered again before exposure. The bacteria were exposed to the radiation in suspensions containing from 1×10^9 to 3.5×10^9 bacteria per cubic centimeter. The suspending physiological solution was non-absorbing for the wavelengths used. This high concentration of bacteria assured a high efficiency in energy utilization, and simplified the calculation of the energy required in producing the desired effects.

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Monochromatic ultraviolet radiation was employed. A quartz-monochromator separated the different wavelengths, the light source being a water-cooled, high pressure, quartz, capillary, mercury vapor lamp (Daniels and Heidt, 1932). The intensity of the radiation was measured with a vacuum thermopile in connection with a high-sensitivity galvanometer, standardized against a Bureau of Standards lamp. There was an exact control of the intensity of the monochromatic radiation entering the exposure cell and of the energy absorbed by the suspension liquid, so that the effective energy absorption per bacterium could be accurately calculated. The material was exposed in a special exposure cell prepared for this work (fig. 1). Both the exposed and the control cells were located in the same temperature-controlled water bath (18°C.), and both were stirred rapidly before and during exposure. These precautions were taken to assure an actual high probability that each organism in the suspension received equivalent quantities of radiation under identical conditions.

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[2] Composition: NaCl, 6 grams; KCl, 0.4 grams; $CaCl_2$, 0.4 grams; water, 1 liter.

For the greater part of the work wavelength ca. 2650 A was used, since it proved most efficient in producing recognizable effects, and it is a band easily isolated in high purity and intensity.

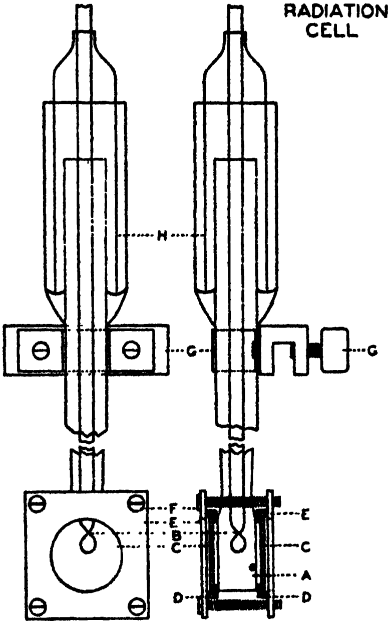

FIG. 1. EXPOSURE CELL

A, cell capacity about 6 cm.^{3}; *B*, stirrer; *C*, quartz windows; *D*, rubber gaskets; *E*, brass frame; *F*, tightening screws; *G*, for attachment to support on temperature tank; *H*, device for liquid seal.

SPECIAL CULTURAL PROCEDURES, OBSERVATIONS AND RESULTS

In the study of bactericidal effects previously reported, equivalent samples from the exposed and from the control cell were transferred, each to a unit quantity of physiological salt solution, shaken thoroughly, and then, after proper dilution, plated in potato gluose agar. It was observed that, in general, the colonies formed by bacteria which had survived irradiation appeared somewhat later than the colonies formed by the control organisms. There was a noticeable lag in development, and while no critical study was made of that phenomenon at the time, it was at least recognized as the first prominent sublethal effect.

A second effect became apparent when it was attempted to obtain a quantitative estimate of the extent of the observed retardation by following comparatively for a few hours the growth curves of irradiated and control organisms during incubation in a liquid nutrient medium. The liquid medium adopted for such incubation series consisted of 99 cc. of salt solution with the addition of 2 to 5 cc. of standard Difco beef broth. The number of colony-forming organisms present at successive time intervals during incubation was determined by the usual agar dilution plates and colony counts. In this work, however, it was essential to obtain an exact measure of the extent of the lag phase[3] and accordingly to have data for closely spaced points along the growth curve, involving a large number of dilution plates during the first few hours of incubation. When plates from such a series were counted, it became apparent that in the exposed series the colony-forming population did not exhibit the characteristics of a typical lag but actually the numbers had apparently increased considerably during the first hour or two of the incubation, provided that the bacteria in the exposed suspension had received such quantities of radiation as to yield a fairly low survival, that is, a high percentage killing. During the same time of incubation the control culture had shown no early increase in population. Thus, there was a second effect manifest from largely-lethal intensities of ultraviolet. We shall in future refer to the first phenomenon, discussed in the preceding paragraph, as the "extended lag phase," and we shall refer to the early apparent increase (in nutrient solution) of surviving organisms which are able to form colonies on nutrient agar as the "apparent initial increase."

These two effects may be made clearer by reference to actual data. Table 1 and figures 2 and 3 illustrate a section of the results of a typical experiment, and will be explained in some detail. First, however, it should be noted that in figure 2 the actual data are plotted, whereas in figure 3 data are all recalculated for comparative effects. The bacteria (in standard salt solution) at a suspension concentration of 3.22 × 10^9 organisms per cubic centimeter were exposed to monochromatic ultraviolet radiation (2650 A) in the temperature bath kept at 18°C., with constant stirring, as indicated. At appropriate time intervals (actually 10, 15, 18, 21, 25, and 30 minutes) after an irradiation intensity such that each organism in the successive samples removed had received respectively 2, 4, 6, 8, 12, 20 × 10^{-6} ergs, accurately measured samples, of $\frac{1}{10}$ cc. each, were removed from the exposure and control cells, diluted as prearranged with physiological salt solution, and finally incubated at 32°C. in 99 cc. salt solution and 5 cc. standard Difco beef broth. A full-size series consisted of six exposures, or runs, and for such a series there were three controls, each uneven run being followed by a control. This also permitted the handling of the dilution plates in groups of three components, which were small enough for rapid work. Then by staggering the pouring of exposed and controls, rather than by pouring the one or the other successively,

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[3] It does not seem practicable for our purpose to distinguish between an approximately stationary phase and a subsequent slow multiplication for a time, since this may all be expressed in the idea of the lag phase of development.

TABLE 1

Survival value of Run 3 = 7.7 per cent and Run 4 = 2.67 per cent

All runs diluted for incubation in 99 cc. salt solution 5 cc. bouillon

COLUMN 1	COLUMN 2	COLUMN 3	COLUMN 4	COLUMN 5	COLUMN 6	COLUMN 7	COLUMN 8
Plating	Run 3, mean of plate counts		Control for runs 3 and 4, mean of plate counts		Run 4, mean of plate counts		Incubation time
	Actual	Recalculated	Actual	Recalculated	Actual	Recalculated	
							minutes
A	230±2.64	100±1.15	322.5±6.2	100±1.92	86±2.8	100±3.26	0
B	287±7.65	123±3.3	325.5±3.84	101±1.19	98±3.57	114±4.5	40
C	334±1.48	145±0.64	326±8.56	101.2±2.66	118.7±3.53	138±4.1	75
D	385±2.31	167±1.65	387±6.1	126.1±1.89	173.3±3.65	201±4.19	117
E	405±6.57	176±2.86	657±41	239±13.4	196.3±3.65	228±4.19	160
F	653±7.93	284±3.44	1800±68	558±20.8	265.7±4.95	308±5.76	213
G	1660±23.5	722±10.2	3970±121	1240±37.7	428±9.56	498±10.2	260
H	7400±36.4	3220±15.9	1300±272	4030±34.2	1390±44.9	1620±52.2	320

a considerable possible error, arising from a longer time-interval between comparable flasks, was avoided.

We may follow, in our typical experiment as an example, a single group of "runs" (a part of the larger experiment), selecting (1) Run 3, (2) the control, and (3) Run 4—the actual data of which were given in figure 2. It should be pointed out that "run" refers to any sample after an interval of irradiation and that the control in this and in certain other cases has received special attention. In keeping with the plan that the control, in selected instances, should contain at the beginning of the incubation series a number of living and of dead organisms of the order of magnitude of the irradiated suspensions, two things have been

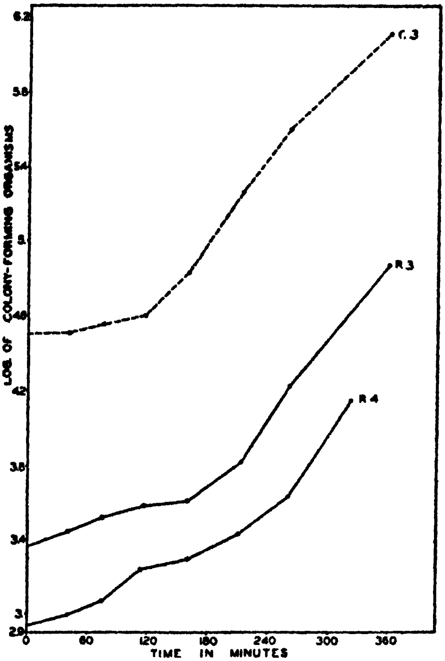


FIG. 2. *Escherichia coli*. Log of actual number of colony-forming organisms surviving in control and in irradiated (2650 A) suspensions, plated out directly after irradiation and at set time intervals. Each organism in Run 3 received 6 × 10^{-6} ergs, a survival value of 7.7 per cent; in Run 4 each received 8 × 10^{-6} ergs, a survival of 2.63 per cent. Broken line, control; continuous lines, Runs 3 and 4.

done. In the first place, it was necessary to dilute the control appropriately with salt solution. (It was also necessary to dilute somewhat the runs from which higher survivals were expected.) Secondly, heat-killed organisms (in other cases radiation-killed) were added in requisite quantity to the control (see also page 30). In Run 3 the number inactivated, as shown by the plate counts, is 92.3 per cent of the bacteria originally able to form individual colonies, while in Run 4 the relative inactivation is 97.37 per cent. At zero time there were in Run 3 of the incubation suspension prepared as indicated 3225 organisms per cubic centimeter, of which 230 were able to form colonies when trans-

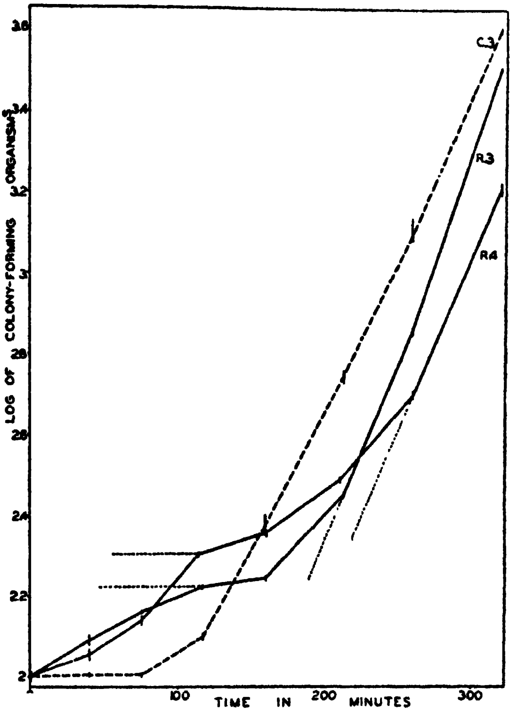


FIG. 3. *Escherichia coli.* After appropriate dilution of the suspensions (control and irradiated) for incubation and zero time platings the log of the recalculated values (see table 1), using the data of fig. 2, are plotted against time. Horizontal lines show extent of "apparent initial increase" (approximately 41 to 75 per cent) and the assurgent dotted lines indicate range of the extended lag phase.

ferred to agar plates, the remainder being radiation-killed, or at least rendered unable to multiply. In Run 4 where there was a total of 3225 organisms per cubic centimeter only 86 were able to form visible colonies. The control suspension contained approximately 3600 organisms, of which the calculation indicates that 322 came from the control lot and were able to form colonies, the remainder representing the dead organisms added.

It is well also to stress the point that the first plating is made as rapidly as possible after the sample has been removed from the radiation cell and transferred to the proper flask for incubation. The subsequent platings are made at appropriate time intervals. A large series may involve 600 plates, so that both speed and accuracy are essential for consistent results. One operator is required at the monochromator and another at the platings. In all cases at least three plates are poured for each dilution, and in most cases one or two dilutions are adequate. The dilution of the bacteria is so estimated that there may be from 80 to 250 colonies per plate. The counts are made after the plates have been incubated for about 48 hours. In our experience, no additional colonies develop with further incubation.

The reliability of the results in this group and the spread of the probable error may be seen in table 1 and figure 3 (recalculated as explained). In the table the results of the successive platings are shown. Column 2 displays the mean of the actual plate count replicates and the probable error. Column 3 is, however, more important in the general overview, affording recalculated values, including the probable error. Recalculation is necessary owing to differences in population at zero time. Accordingly the recalculation is effected by considering the number of individuals in each run and each control independently as 100 at zero time. The formula for this recalculation is $m \times 100/n = p$, in which m is the uncorrected value (mean of plate counts) of any run or control at any time interval; n is the uncorrected value (mean of plate counts) at zero time; and p is the percentage value by comparison with the value at zero time. The purpose of these values is, of course, a direct comparison of growth rates. By comparison then Columns 4, 5, 6, and 7 give corresponding values for the control (Columns 4 and 5) and for Run 4 (Columns 6 and 7). The elapsed time intervals of the platings are shown in Column 8. In figure 2 the log of the mean of the actual colony number per plate is plotted against time of incubation, whereas in figure 3 the corrected values are on the basis of 100 for each run and for the control. The value of such recalculations can be recognized by comparing figures 2 and 3.

The regularity of the normal growth curve is enhanced by the incubation of the cultures in a well-stirred water bath. Incubation in air did not yield such concordant results. The temperature of incubation was 32°C., which is not the optimum for these organisms; but the lag phase was extended at this temperature and the growth rate was such as to permit the determination of several points on the curve before the log phase was evident.

Nevertheless, it should be stressed that in order to locate such points, and additional ones, properly spaced on the growth curve, and thus bring out the characteristic growth effects, it is requisite even under the most favorable conditions to organize carefully the plating procedure and to have available an abundance of sterile media as well as vessels of every sort required. Especially essential is a large supply of perfectly delivering standardized pipettes, since no pipette may be used more than once without cleaning.

Referring again to figure 3, graphing the data discussed in the preceding paragraphs, the "apparent initial increase" and the "extended lag phase" are clearly indicated. Using Fisher's (1924) method for significant differences of small samples, the so-called "t" test, it is shown that both of these growth curve modifications referred to are statistically safe. An example of such calculations is offered in another publication (Hollaender and Claus, 1937).

Experiments with *Serratia marcescens* have yielded results confirming the two growth effects observed. In figure 4 are displayed growth curves of this species representing a series of three exposures and two controls. Both the apparent initial increase in the number of colony-forming organisms and the extended lag phase are obvious by comparison of the behavior of the exposed and the control cultures. In this case the exposed cultures in Runs 1, 2, and 3 represent respectively survival values of 41, 11, and 2.6 per cent. Note the apparent initial increase at the lower survival value. No other species of bacteria have been tested, since the work with *E. coli* alone has required a considerable number of experiments necessitating handling of many thousand plates; and it has seemed more desirable for the present to study this organism intensively than to obtain limited data with an assortment of organisms.

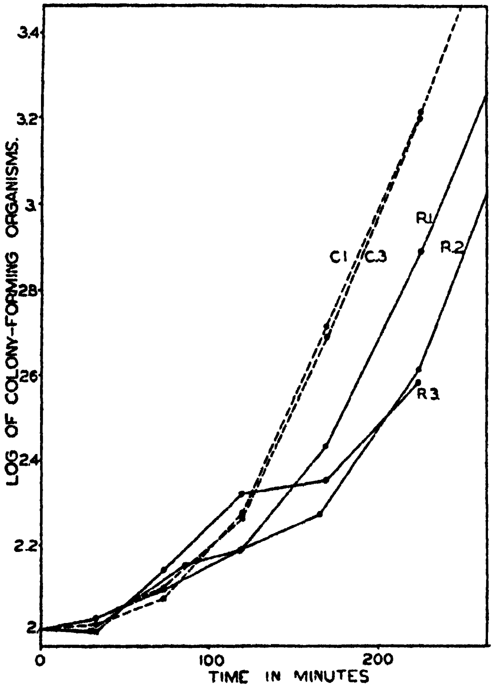


FIG. 4. *Serratia marcescens*. Growth curves of control and irradiated cultures recalculated as in fig. 3 from the original data. Complete indications in text.

INFLUENCES AFFECTING THE APPEARANCE OF THE GROWTH PHENOMENA OBSERVED

Selection for the experimental work of cultures grown from 15 to 39 hours at 32°C. has been made on the basis of experience, since the age of the culture affects the response to radiation. In figure 5 are given, as examples, growth curves for a 15-hour and a 48-hour culture. Cultures younger than 15 hours gave a lag phase too short to make possible the effective determination of an adequate number of points in this part of the curve.

Washing the bacteria has no direct effect on the appearance of the special phenomena here considered, although somewhat less energy is required with washed organisms to produce these effects, and this is in accord with earlier findings that the efficiency of bactericidal action is increased slightly after washing. That no detectable effect is produced on the suspending liquid under the conditions applying is of much importance. Special tests were made by irradiating the salt solution and observing comparatively the growth properties of bacteria in it and in a control,

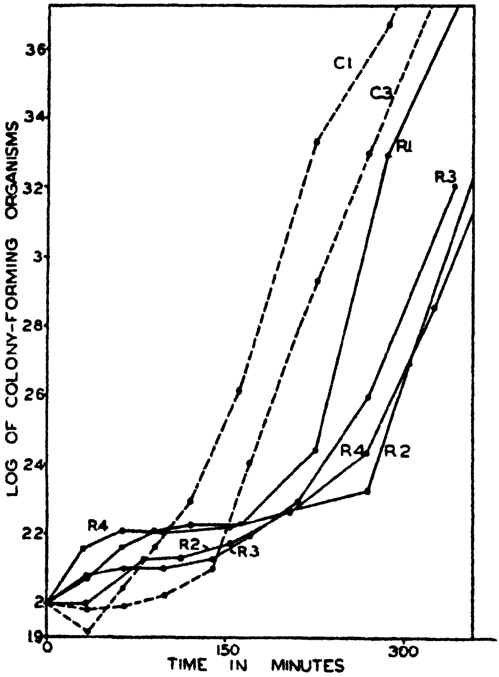


FIG. 5. *Escherichia coli*. Growth curves of control and of irradiated cultures, recalculated as in fig. 3 from the original data. *R.1* and *R.2* were exposed to different energy values (*R1* < *R2*); these and *C1* (control) were from a 15-hour culture, while *R.3*, *R.4*, and *C.3* were from a 48-hour culture, but treated in a manner identical with the first group.

after the addition of sufficient beef broth to afford growth. This refers only to wavelengths and energies used in this investigation.

In order to display the relation of the effects here discussed to bactericidal action, figure 6 is introduced, showing the results in the form of a typical killing curve at 2650 A. The logarithm of the survival value is plotted against the energy necessary to kill one organism. This graph is divided into four sections, *A*, *B*, *C*, and *D*. The extended lag phase will appear in sections *B*, *C*, and *D*, the apparent initial increase in section *C* only.

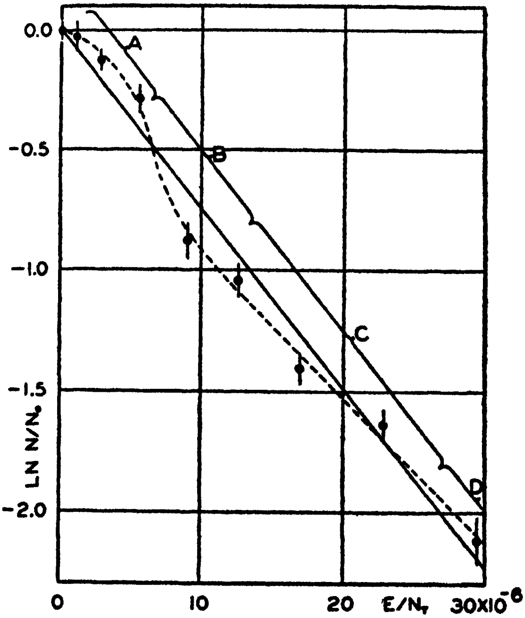
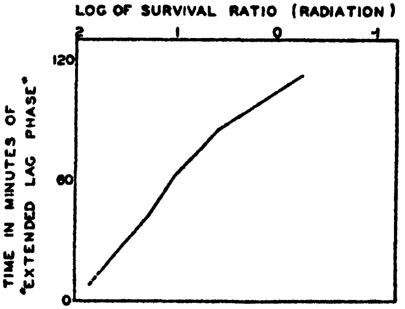


FIG. 6. *Escherichia coli*. This figure redrawn from Hollaender and Claus (1936), and extended, shows the relation of the apparent initial increase and of the extended lag to bactericidal action. While the extended lag appears in sections *B*, *C*, and *D*, the apparent initial increase is in section *C* alone.

Quantitative response is obtained, depending on the energy applied. As a final test of the types of quantitative response several complete experiments were made. In these, at least six exposures and three controls were included. During the irradiation, successive samples were removed to afford increasing energies, and growth curves for each energy level were followed.

It was then found that the apparent initial increase is governed very closely by the percentage killing (and no doubt by the general procedure to a lesser degree). A concrete case will illustrate this point. If 1,000,000 are irradiated and 999,900 are killed the surviving 100 organisms may not show the initial effect, certainly not in pronounced form; but if 980,000 are killed, the surviving 20,000 will show probably the highest possible increase. On the other hand, when 999,900 are killed the surviving 100 will show the most pronounced extended lag. Two figures (7a and 7b) illustrate the point discussed.



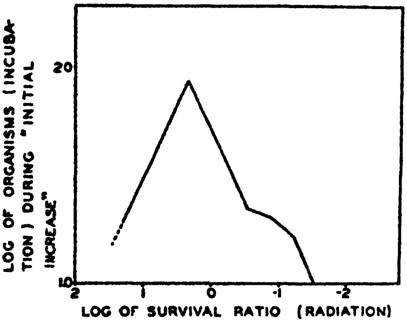


FIG. 7a. *Escherichia coli.* This graph shows the time of the extension of the lag phase plotted against the log of the survival ratio; it illustrates the time elapsed after the controls have started to divide before the irradiated bacteria go into their *normal* growth phase, and the dependence of the extension on the amount of radiation used.

FIG. 7b. *Escherichia coli.* This curve shows the magnitude of the initial increase, that is, the increase (log) of colony-forming organisms in the irradiated suspensions plotted against the log of the survival ratios.

The maximum display of the apparent initial increase occurs at around 95 per cent killing (actually 94 to 98 per cent), and decreases with lower or with higher survival ratios, none of this effect being observable in sections *A*, *B*, and *D* of the inactivation curve (fig. 6). Its occurrence within so limited a set of survival values has no doubt obscured this phenomenon. On the other hand, the extended lag phase will appear over a relatively wide range of values, although more pronounced when survival is down toward one percent. At very low survival values the extended lag phase is, in fact, recognizable even in the delayed appearance of the colonies on the agar plate.

Perhaps it is necessary to defend or explain that part of our procedure in which dead (irradiation-killed or heat-killed) bacteria are added to the control groups attempting to simulate exposure conditions, with the possibility of establishing some causative relations of the irradiation to the observed effects, especially in the case of the apparent initial increase in the number of colony-forming organisms. There was the possibility (1) that materials diffusing out of the radiation-killed (as well as out of radiation-surviving) bacteria might produce a "stimulating" effect, or (2) that differential effects might be produced as a result of differences in number of living, dividing organisms per cubic centimeter in suspensions containing control or irradiated bacteria.

Each of these possibilities has been investigated and eliminated as a plausible explanation or a source of error in the results. In various experiments bacteria killed by irradiation or bacteria killed by heat treatment have been added to the controls in number approximately corresponding to the number of dead organisms in the irradiated suspensions. In no case was any effect, of the order sought, apparent with the quantities added. The energy used for killing the bacteria by irradiation was no more than was necessary to attain 100 per cent efficiency, since it is to be assumed that at very high energies changes might be induced in the dead bacteria which might have either a beneficial or an injurious effect (Blank and Arnold, 1935).

The concentration of the nutrient beef broth seemed not to have been a very important factor in the appearance of the effects discussed. A low concentration nutrient medium was selected for convenience. The pH of the medium had, as far as tested, no differential effect on control and irradiated suspensions. The reality of the effects described can be seen both from the experiments in which each step has been done in quadruplicate (Hollaender and Claus, 1937, p. 80) and also from the tests previously made on the delay of the two new phases as induced by keeping the irradiated and the control suspensions, after irradiation, in a bath at 0°C., for a certain time interval (l. c., p. 87). The final yield, after extended time, was apparently the same in the flasks containing the control as in that containing the exposed organisms. Apparently control and exposed organisms grow until the nutrient material is used up, or until toxic substances formed make further growth impossible. Nevertheless, additional work on this point is very desirable.

It is not infrequently stated that in making a transfer from an old culture of bacteria to a fresh medium the lag or resting phase may be shortened by using as inoculum a relatively large number of organisms per cubic centimeter rather than a small number (cf. Buchanan and Fulmer, 1928, vol. 1). While relative quantity of inoculum could hardly play a rôle in the experiments here described, the matter has received attention and precautions were taken. The population of the inoculum was so adjusted that the number of colony-forming organisms in each incubation flask was comparable at the beginning; thus, if the indications were that 80 per cent of the organisms had been killed by irradiation, greater dilution was made than if the inactivation had affected 98 per cent.

TABLE 2

MEAN NUMBER OF COLONIES EXPOSED	MEAN NUMBER OF COLONIES CONTROL	EXPOSED PLUS CONTROL CALCULATED	EXPOSED PLUS CONTROL EXPERIMENTAL
124	86.5	210.5	236
139	81.7	220.7	238
202	94	296	258
230	94	324	272
250	147	397	402
300	190	490	506
93	80.2	173.2	156.3
88	85	173	170
143	90	233	196
147	93	240	210
192	152	344	335
520	482	1002	802

Table 2 exhibits the results of two experiments in which, immediately following the irradiation interval, two separate incubation series were set up, and parallel with these a mixed suspension of irradiated and control organisms was arranged. With the development from zero time the number of colony-forming organisms was determined in the usual manner, that is, by dilution plates made at set intervals, and the counts were also made as usual. In column 1 the means of the plate counts derived from the irradiated suspension are displayed and in column 2 corresponding figures for the unirradiated control. The data in column 3 have been obtained by adding columns 1 and 2. These last figures represent "calculated" values for the mixed solution in the circumstance that irradiated organisms mixed with control organisms do not recognizably alter the growth rate of the control. In column 4 are given the means of the actual plate counts furnished by the mixed suspension prepared as previously indicated. The agreement between the corresponding figures in columns 3 and 4 is satisfactory, suggesting the absence of any effect of irradiated upon unirradiated organisms under the conditions of these experiments.

DISCUSSION

It should perhaps be made clear that the energies employed in this work are far beyond the order of magnitude of those considered to be involved in the alleged "mitogenetic radiation." In the study here reported, the energies are in the neighborhood of 2000 ergs per $cm.^2$ per sec., whereas the energy claimed for mitogenetic radiation is 10^{-9} to 10^{-11} ergs per $cm.^2$ per sec., so that it seems unnecessary to take this point further into consideration.

The effects reported here are on the organisms; and, as previously indicated, they are not the result of changes in the suspending liquid.

Since growth properties exhibited by the organisms surviving irradiation at the intensities here considered, in comparison with the control organisms, constitute the chief interest in this work, it seems well to emphasize certain conditions of the culture technique. After irradiation, suitable dilutions of the irradiated and control suspensions are made in a beef broth medium. Zero-time dilution cultures are made on agar plates. Samples from the (1) irradiated and (2) control are then incubated as parallel series in the broth medium and platings are made at various intervals. All conditions are similar for the two series.

It may be well to consider separately the provisional interpretations of the "extended lag" and of the "apparent initial increase." The most reasonable explanation of the "extended lag" phase may be found perhaps in the assumption that the bacteria have been somewhat injured during the process of irradiation, but not sufficiently to impair permanently their ability to divide. Whether the injury is produced by direct or indirect effects cannot be decided at the present stage of this investigation. Any explanation which is adequate must take into consideration the fact that the "extended lag" is not eliminated by any preceding "early apparent increase." It is well to recall also that the addition of irradiated to control organisms affords no evidence that the control organisms in the mixture are affected by materials transferred with the irradiated bacteria. It is also possible that the bacterial cell contains enzymes or moderately photosensitive compounds which respond to such quantities of energy as are specified in this work, despite the fact that the destructive effects of ultraviolet radiation on enzymes ordinarily require considerably more energy than is necessary to induce the "extended lag." That this lag extension has not earlier received more attention is probably a result of the methods of investigation employed, these methods making it difficult, if not impossible, to recognize effects other than those which are purely lethal (cf. footnote p. 20). From a purely biological viewpoint, the occurrence of a recovery time after injury is reasonable.

There seems to be, unfortunately, no equally reasonable explanation of the early apparent increase. In the several paragraphs following, an attempt is made to analyze certain of the possibilities.

Clumping, as a result of irradiation, and de-clumping, after transfer to broth, came up as a possibility; but the idea is not supported by any evidence we have been able to obtain from the results of vigorous shaking or from microscopic study.

The problem of the microscopic study of the phenomenon is frankly a difficult one. Evidence from microscopic study would be more easily obtained with other organisms. The difficulty of establishing such a point by a microscopic count of cells may be made clear from the following. If, in an irradiated suspension of unit quantity, there is a total of 1000 individuals, 100 of which increase during the early incubation period to 150 or 200, the original 1000 becomes 1050–1100, and this represents a change in total cell number of from 5 to 10 per cent. Such an increase cannot be established accurately by means of microscopical cell counts. Furthermore, microscopical counts are less accurate in very low concentrations, whereas the initial increase comes out more clearly in high dilution of the incubated suspension. We shall postpone further discussion on this basis until our studies on yeast, to be reported later, are available.

It appears that in the suspension of bacteria employed in the incubation series, there are about 5 to 10 per cent of double cells. These are present in spite of vigorous shaking and filtering, but the segregation of these would not account for the observed increase of 30 to 40 per cent or more.

The early apparent increase in the number of colony-forming organisms might seem to arise as a result of a prompt recovery of a certain percentage of cells, followed later by the characteristic lag which leads into division at the normal rate. This again would ignore the fact that "recovery" should then be manifest when the zero-time plating is made. There is the further fact that the extended lag phase is evident with 70 per cent survival; whereas, the apparent initial increase appears around 20 per cent survival, and is more pronounced with 8 per cent or less. It would appear that simple recovery should be most pronounced at the high survival values, since there the cells would be least damaged.

In almost every contribution on the bactericidal effect of ultraviolet radiation there is some mention of the possibility of growth "stimulation" by means of these wavelengths (see the review by Duggar, 1936). In most of these papers the evidence is interpreted as negative, but there are exceptions, such as those of

Browning and Russ (1917). However, we agree with Bachem and Dushkin (1933) in the interpretation that the so-called stimulation in this case was merely the growth of larger colonies (with more space and available nutrients) in areas of exposed plates where survival was not high. Bovie (1916) suggested (without explanation) that partial decomposition of the protein "may stimulate the cell." Coblentz and Fulton (1924) report as follows: "There is some evidence that organisms are stimulated in growth when exposed to low intensity ultraviolet radiation." No experimental data were included. Certain morphological modifications resulting from irradiation have been observed by Gates (1933). In this short note, without special data, referring to "degenerative" changes in organisms strongly irradiated but not killed, he lists "gradual degeneration," division into "large or small units, which then degenerate," and "daughter cells may pinch off at one end and multiply rapidly to colony formation."

The data presented indicate that a significantly small surviving population from a given light intensity exhibit on incubation an "apparent initial increase." Is this "increase" the result of early cell multiplication? If the individuals divide promptly on incubation, why do they not continue, in a nutrient medium, to divide rather than pass into a typical lag? If cell division is assumed, what is a plausible suggestion regarding the mechanism?

From the observations recorded in this paper little or no support is given the possibility that the initial increase is either a recovery phase or a loss of clumping property; rather the evidence seems to point to the hypothesis—not yet verified by all possible means of attack—that the "apparent initial increase" described is actually a multiplication of cells. Any hypothesis regarding the mechanism of the extended lag phase should take into consideration the probability that over and above the intracellular products that may determine the normal lag there is doubtless a further addition of inhibiting substances as a result of irradiation. All these, however, must play a secondary role as long as the division-promoting substance is present in sufficient concentration.

This work was conducted with the support of the Committee on Radiation, Division of Biology and Agriculture, National Research Council.

SUMMARY

1. Two effects of sublethal doses of ultraviolet radiation were observed; (*a*) an apparent initial increase in number of colony-forming organisms, and (*b*) an extension of the lag phase.

2. Methods and procedures were developed for the irradiation process and the recognition of the sublethal effects.

3. These effects have been illustrated by typical experiments.

4. The influence of a number of factors on the appearance of the phenomena have been followed, e.g., treatment of the organisms before, during, and after irradiation, giving special attention to the reproducibility of the effects.

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THE CLASSIFICATION OF ACID-FAST BACTERIA. II

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Received for publication December 8, 1937

In a previously reported attempt (Gordon, 1937) to find standards for the classification of the rapidly-growing, non-pathogenic mycobacteria, we presented the results of a cultural study of a collection of 252 strains from soil, plants, human and animal tissues and secretions. Eighty per cent of the strains were readily separated into three groups of closely-related cultures. The remaining 20 per cent of the strains, which differed from the above and in most cases from each other, were arbitrarily distributed among the three major groups as sub-groups. Since 84 per cent of the cultures of the collection had been isolated from soil by means of Söhngen's technique, there arose the question of whether cultures from sources other than soil would fall into the same groups and whether the standards previously set would be adequate. It seemed possible that some of the unrelated, single strains of our soil collection might be represented in large numbers in an assemblage of cultures from other sources and isolated by other methods.

Seventy-nine additional strains of saprophytic acid-fast bacteria have been studied by the same methods described in our previous report. The numbers and sources of the cultures are recorded in table 1. The fish strains were isolated on glycerinated egg medium; the soil and water cultures were obtained by Söhngen's technique (1913).

EXPERIMENTAL

Two of the three major groups, first devised by Thomson (1932) for the classification of the saprophytic acid-fasts and employed in our previous study, were represented in the more recent collection. None of the cultures were of the *Mycobacterium phlei*, or Group III, type. Seventeen strains, fifteen of which are of the *Mycobacterium smegmatis* type, were placed in Group I. The remaining cultures were assigned to the more heterogenous Group II.

The cultures have been grouped according to a plan which differs slightly from our first arrangement. In Group I, the two sub-groups, which previously had been separated on the basis of arabinose utilization, were separated on the basis of utilization of arabinose, sorbitol, galactose, trehalose, mannitol, and fructose. In Group II, a new sub-group, IIc, was created for the disposal of those cultures which did not utilize any of the carbohydrates employed. The altered plan and the grouping of the new cultures are as follows:

TABLE 1

NUMBER	CULTURE	SOURCE
11	Human and rat leprosy	American type culture collection
1	Bovine tissue	E. G. Hastings
1	Sputum	L. V. Gardner
1	Saprophytic strain*	Dairy department, Cornell University
10	Spontaneous tuberculosis of killifish (*Platypoecilus maculatus*)	J. A. Baker
39	Water in killifish aquaria	J. A. Baker
16	Local soil	R. E. Gordon

* This culture came to us labelled "The original Koch strain of human tuberculosis." Acting upon the recommendation of Hastings and McCarter (1932), who inquired into the history of and studied several so-called saprophytic human and bovine tubercle bacilli, we have not used this name.

Group I

Fails to survive 60°C. for one hour.
Grows at 47°C.

a. Utilizes arabinose, sorbitol, galactose, trehalose, mannitol, and fructose. Fifteen strains similar in appearance and cultural reactions.

b. Unable to utilize all the above carbohydrates. Two strains differing in appearance and cultural reactions from the above cultures and from each other.

Group II

Fails to survive 60°C. for one hour.
Does not grow at 47°C.

a. Unable to utilize sorbitol.
    1. Unable to utilize arabinose. Forty strains similar in appearance and cultural reactions. Two dissimilar strains.
    2. Utilizes arabinose. Two similar strains.
b. Utilizes sorbitol. Eight dissimilar strains.
c. Unable to utilize any of the carbohydrates tested. Ten similar strains.

Group Ia. The strains assembled in this group produced a characteristic growth and color change. The growth was rapid, spreading, and wrinkled. At first, the growth was a dull, creamy white, but after four to six weeks at room temperature the cultures became a deep yellow or orange. All of the test carbohydrates, except sucrose, were utilized. Of the fifteen cultures placed in this group, ten were soil isolations; one strain had been isolated from sputum of a tuberculosis patient; another strain was the so-called saprophytic tubercle culture; and the remaining three strains bore the name, *Mycobacterium leprae.* Two of the latter had been isolated from leprous rats; the third was the culture of Levy-Kedrowski.

Group Ib. This group was created for the disposal of cultures which grew at 47°C., but were not of the *Mycobacterium smegmatis* type. Four dissimilar cultures, two leprae strains and two soil isolations, had been assigned to this group in the previous study. Two more cultures, the leprae strain of Lombardo-Pellagrine and the isolation from bovine tissue, were added from the new collection. The resulting six cultures were dissimilar in appearance and biochemical reactions.

Group IIa₁. The predominating culture type of this group produced a creamy white growth which did not change color with aging. It was either rough or smooth in type; it utilized fructose and, in most instances, trehalose and mannitol. Forty cultures, one a soil isolation and thirty-nine water strains, were assigned to this group.

Another type placed in this group showed the same biochemical characteristics as the predominating type, but produced a yellow pigment. Our first collection contained ten of these yellow pigmented strains, and our second collection had one, a soil isolation.

The remaining dissimilar strain assigned to this group, *Mycobacterium leprae* of Brinkerhoff, is a smooth, pale yellow culture which utilized mannitol, trehalose, fructose, and sucrose. It was similar in appearance and biochemical reactions to our Duval leprae strain previously included in this group.

Group IIa$_2$. The two smooth, salmon-pink, soil cultures placed in this group utilized arabinose, galactose, trehalose, mannitol, and fructose. These cultures resembled none of the seven dissimilar cultures previously assigned to this group.

Group IIb. The eight cultures assembled in the group represent six different types. A smooth, yellow soil isolation and the leprae strain of Needham appeared to be identical in appearance and cultural reactions. They utilized all carbohydrates tested and resembled two soil isolations previously described.

A rough, creamy white, soil culture utilized sorbitol, mannitol, trehalose, and fructose and was similar to three soil isolations previously included in this group.

The remaining five leprae strains were nearly identical in biochemical characteristics, but varied greatly in appearance. The smooth, salmon-pink, leprae culture of Levy-Chrome was apparently identical with the leprae strains of Brinkerhoff and Clegg previously studied. The red-pigmented culture of Rost-Williams resembled a soil isolation of our former collection. The rat leprosy strain of McCoy resembled the leprae strain of Elly previously included in this group. The two pale-yellow, pigmented cultures of Currie and Barry seemed closely related, if not identical, with each other.

Group IIc. The ten yellow pigmented cultures isolated from spontaneous tuberculosis of killifish (*Platypoecilus maculatus*) utilized none of the carbohydrates employed. They would not grow at 37°C., nor on Söhngen's medium. They were not identical with any culture in our former collection, although seven of our first cultures, including *Mycobacterium cheloni* and *Mycobacterium schlangen,* did not utilize any of the seven carbohydrates. These seven cultures were previously placed in Group IIa$_1$. It seemed advisable to create a new subdivision of Group II and to assign to it these seventeen comparatively inactive cultures.

TABLE 2

Cultural characteristics and grouping of our collection of saprophytic, acid-fast cultures

GROUP	NUMBER OF CULTURES	SURVIVAL AT 60°C. FOR 60 MINUTES	GROWTH AT 47°C.	GROWTH ON MERRILL'S MEDIUM WITH VARIOUS CARBON SOURCES							GROWTH ON DORSET'S SYNTHETIC MEDIUM, NO GLYCEROL	ALKALI PRODUCTION IN LITMUS MILK	NITRATE REDUCTION
				Sorbitol	Arabinose	Galactose	Trehalose	Mannitol	Fructose	Sucrose			
Ia	120	−	+	+	+	+	+	+	+	−	−	+	116 + 4 −
Ib	6*	−	+	±	±	±	+	±	+	±	−	+	±
IIa$_1$	106	−	−	−	−	−	±	±	+	−	−	+	±
	11	−	−	−	−	−	+	+	+	−	−	+	+
	6*	−	−	−	−	−	±	±	+	±	−	±	±
IIa$_2$	9*	−	−	−	+	±	±	±	+	±	−	±	±
IIb	16*	−	−	+	−	−	+	+	+	±	−	+	±
	10*	−	−	+	±	±	±	±	+	±	−	+	±
IIc	10	−	−	−	−	−	−	−	−	−	−	sl	±
	7*	−	−	−	−	−	−	−	−	−	−	±	±
IIIa	29	+	+	+	+	+	+	+	+	−	±	+	±
IIIb	1	+	+	sl	−	sl	+	+	+	−	−	sl	+

* Dissimilar cultures.

A compilation of the data on all the cultures we have studied is presented in tables 2 and 3. The new plan of culture-grouping affected the arrangement of the cultures first studied only slightly, the one change being the assignment of seven strains previously

TABLE 3

Source and grouping of our collection of saprophytic, acid-fast cultures

GROUP	NUMBER OF CULTURES	NAME OF CULTURES	SOURCE	ISOLATED BY
Ia	1	*M. smegmatis*	Smegma	Alvarez and Tavel
	2	*M. graminis*	Plant dust	Moeller
	2	*M. stercusis*	Cow manure	Moeller
	1	*M. berolinense*	Butter	Rabinowitch
	1	*M. paratuberculosis*		Binot
	1	*M. pseudoperlsucht*		
	1	Grassberger's bacillus	Butter	Grassberger
	1	*B. aus Nasenschleim*	Nasal exudate	Karlinski
	1	Traum's bacillus	Bovine lymph gland	Traum
	1	*M. leprae*	Human leprosy	Levy-Kedrowski (A.T.C.C.)
	2	*M. leprae*	Rat leprosy	Am. Type Cul. Collection
	1	Saprophytic Koch strain		
	1	Unnamed	Sputum	Gardner
	104	Unnamed	Soil	Cornell University
Ib	1	*M. leprae*	Human leprosy	Clegg
	1	Kat #352	Human leprosy	Carpenter
	1	*M. leprae*		Lombardo-Pellagrine (A.T.C.C.)
	1	Unnamed	Bovine tissue	Hastings
	2	Unnamed	Soil	Cornell University
IIa$_1$	1	*M. ranae*	Frog's liver	Kuster
	1	*M. thamnopheus*	Garter snake	Aronson
	1	*M. leprae*	Human leprosy	Duval
	1	*M. leprae*	Human leprosy	Brinkerhoff (A.T.C.C.)
	39	Unnamed	Water	Cornell University
	80	Unnamed	Soil	Cornell University
IIa$_2$	1	*M. marinum*	Fish	Aronson
	1	Lymph gland acid-fast	Bovine lymph gland	Hastings
	7	Unnamed	Soil	Cornell University
IIb	1	*M. leprae*		Lister Institute #518
	1	*M. leprae*		Elly
	1	*M. leprae*	Human leprosy	Brinkerhoff
	1	*M. leprae*	Human leprosy	Clegg
	1	*M. leprae*	Rat leprosy	McCoy (A.T.C.C.)
	1	*M. leprae*	Human leprosy	Currie (A.T.C.C.)
	1	*M. leprae*		Barry (A.T.C.C.)
	1	*M. leprae*	Human leprosy	Rost-Williams (A.T.C.C.)
	1	*M. leprae*	Human leprosy	Levy-Chrome (A.T.C.C.)
	1	*M. leprae*		Needham
	1	Wong strain	Human leprosy	Carpenter
	1	Unnamed	Skin lesion of calf	Daines
	14	Unnamed	Soil	Cornell University
IIc	1	*M. chelonei*	Turtle's lung	Friedmann
	1	*M. schlangen*	Snake	Sibley
	1	Bayne-Jones' acid-fast	Human lung	Bayne-Jones
	1	Plum's bacillus	Milk	Plum
	1	Guernsey heifer acid-fast	Bovine tissue	Hastings
	2	Unnamed	Soil	Cornell University
	10	Unnamed	Fish	Cornell University
IIIa	2	*M. phlei*	Timothy grass	Moeller
	1	Unnamed	Hen's spleen	Cornell University
	2	Unnamed	Bovine lymph gland	Cornell University
	1	Zeissig's bacillus		Cornell University
	22	Unnamed	Soil	Cornell University
	1	Unnamed	Bovine skin lesion	Cornell University
IIIb	1	Hog's skin bacillus	Hog's skin	Buckley

placed in Group IIa$_1$ to the new sub-group IIc. Of the total 331 cultures, 276 strains fell into four groups of closely related cultures, while the remaining 55 strains, or seventeen per cent of the total number, were temporarily distributed among the various sub-groups.

TABLE 4

Grouping and cultural characteristics of leprae strains

NAME OF ORIGIN	SURVIVAL AT 60°C., FOR 60 MINUTES	GROWTH AT 47°C.	GROWTH ON MERRILL'S MEDIUM WITH VARIOUS CARBON SOURCES							GROWTH ON DORSET'S SYNTHETIC MEDIUM ON GLYCEROL	REDUCTION OF NITRATES	ALKALI IN LITMUS MILK	GROWTH ON SÖHNGEN'S MEDIUM
			Sorbitol	Arabinose	Galactose	Trehalose	Mannitol	Fructose	Sucrose				
Group Ia:													
M. leprae, rat 2, A.T.C.C	−	+	+	+	+	+	+	+	−	−	+	+	+
M. leprae, rat 1, A.T.C.C	−	+	+	+	+	+	+	+	−	−	+	+	+
M. leprae, Levy-Kedrowski	−	+	+	+	+	+	+	+	−	−	+	+	+
Group Ib:													
M. leprae, Clegg I	−	+	+	−	+	sl	+	+	−	−	+	sl	+
M leprae, Lombardo-Pellagrine.	−	+	+	−	−	+	+	+	−	−	+	+	+
Human leprosy, Carpenter	−	+	+	−	−	+	+	+	−	−	+	+	+
Group IIa$_1$:													
M. leprae, Duval	−	−	−	−	−	+	+	+	+	−	−	sl	+
M. leprae, Brinkerhoff	−	−	−	−	−	+	+	+	+		−	+	+
Group IIb:													
M. leprae	−	−	+	−	−	+	+	+	−	−	+	sl	+
M. leprae, Elly	−	−	+	−	−	+	+	+	−	−	+	sl	+
M. leprae rat, McCoy.	−	−	+	−	−	+	+	+	−	−	+	+	−
M. leprae, Brinkerhoff II	−	−	+	−	−	sl	+	+	−	−	+	sl	+
M. leprae, Clegg II	−	−	+	−	−	+	+	+	−	−	+	sl	+
M. leprae, Levy-Chrome	−	−	+	−	−	+	sl	+	−	−	+	sl	+
M. leprae, Currie	−	−	+	−	−	+	sl	+	−	−	+	sl	+
M. leprae, Barry	−	−	+	−	−	+	+	+	sl	−	−	sl	+
M. leprae, Rost-Williams	−	−	+	−	−	+	+	+	+	−	+	+	+
M. leprae, Needham	−	−	+	+	+	+	+	+	+	−	−	+	−
Human leprosy, Carpenter	−	−	sl	−	−	−	−	+	−	−	+	+	−

Our entire collection now contains nineteen strains named *Mycobacterium leprae*. These cultures show considerable variation both in appearance and cultural characteristics (table 4). As previously stated, some of them (five) could not be distinguished from certain soil isolations. Ten strains (two pale-yellow pigmented, four deep yellow, and four salmon-pink) of the nineteen utilized sorbitol, but not arabinose or galactose, and did not grow at 47°C. Only seven of our soil isolations showed this combination of reactions, but they did not resemble any of the leprae strains in appearance. Attempts to separate the leprae cultures more definitely from the soil strains have been unsuccessful.

SUMMARY

Seventy-nine acid-fast cultures from cases of human and rat leprosy, bovine tissue, sputum, fish, water and soil were separated into two major groups according to previously set standards. Sixty-five cultures (eighty-two per cent) were readily assigned to closely related groups. The remaining fourteen strains were arbitrarily distributed among four heterogeneous sub-groups.

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A STUDY OF GELATIN DIGESTION BY BACILLUS SUBTILIS

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Received for publication December 23, 1937

The following research was started through the observation that the rate of gelatin decomposition by *Bacillus subtilis*, measured by formol titration, continued to increase for at least 5 days after the cells had reached their maximal number. This behavior which differs fundamentally from that of sugar fermentations was finally explained by the fact that decomposition of gelatin is brought about by an extracellular enzyme while sugar fermentations are due to endo-enzymes.

The rate of any fermentation is proportional to the number of enzyme molecules. In fermentations brought about by endo-enzymes, such as lactic, alcoholic and colon fermentations, the enzyme concentration is proportional to the number of cells, for it seems reasonable to assume that the average enzyme content per cell is fairly constant. Thus, when the maximal number of cells has been reached, the enzyme concentration can increase no more, and the maximal rate of fermentation is reached at this point. One of the authors (Rahn, 1910) has worded this somewhat differently: "The point of inflection (of the curve of products plotted against time) indicates the moment when the maximum number of bacteria is present." Accumulation of products may retard the rate even before the maximal number of cells has been reached, as e.g., in the lactic fermentation, figure 1.

This rule does not apply to extracellular enzymes. They are comparable to secreted metabolic products of the cell. They are produced either as long as the cell lives, or until old age retards all metabolism, or until the enzyme concentration of the medium prevents further enzyme formation. Such enzymes are very probably secreted even after multiplication of cells has ceased. While the cell number begins to decline, the enzyme concentration still increases, and the rate of decomposition may become more rapid for some time.

METHOD

Three hundred and fifty cubic centimeters of a 5 per cent gelatin solution, inoculated with 5 cc. of a broth culture of *Bacillus subtilis*, was incubated at 37°. Plate counts were made daily until the cell numbers began to decrease, and formol titrations were made frequently until constancy.

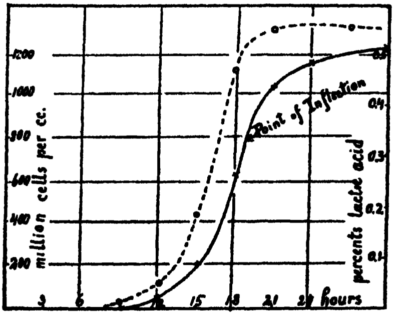


Fig. 1

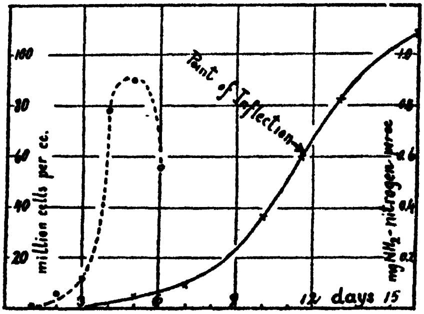


Fig. 2

Fig. 1. Acid Formation by Streptococcus lactis
Full line = per cents of lactic acid; dotted line = cells per cubic centimeter.

Fig. 2. NH_2 Formation by Bacillus subtilis
Full line = milligrams NH_2-nitrogen per cubic centimeter; dotted line = cells per cubic centimeter.

The formol titration was carried out by diluting 10 cc. of the culture with 10 cc. of water. This mixture was titrated with NaOH and phenolphthalein to a deep-red endpoint. Ten cubic centimeters of formalin adjusted to the same endpoint was added, and after the reaction had taken place for one minute, the sample was titrated again to the same endpoint. The difference in titration values gave the milligrams of amino and ammonia nitrogen per cubic centimeter of culture.

RESULTS

Table 1 and figure 2 show a typical result. The cells reach their maximum on the 5th day while the amount of gelatin decomposed per day increases until after the 10th day, which is quite evident from the upward trend of the products curve. The calculation of products per cell per hour which, in case of lactic or alcoholic fermentation, gives at first a constant rate and later rapidly decreasing values, shows here an increase until the 12th day.

The assumption made above that the enzyme is extracellular needs proof for it may be that the partially decomposed gelatin diffuses into the cells, and the actual formation of NH_2 groups is an intracellular process after all. Probably, part is actually intracellular, but this fraction cannot be large as the following experiment shows.

TABLE 1

Amounts of amino and ammonia nitrogen produced from gelatin

TIME	PLATE COUNT	$NH_2 + NH_3$ NITROGEN PER CUBIC CENTIMETER OF CULTURE		
		Total	Daily increase	Per cell per hour
days	*cells per cc.*	*mgm.*	*mgm.*	*10^{-10} mgm.*
0	24,000	0.304	0	
1	520,000			
2	6,300,000	0.311	0.0035	0.081
3	11,000,000			
4	78,000,000			
5	90,000,000	0.352	0.0136	0.18
6	56,000,000			
7		0.395	0.0215	0.13
10		0.661	0.0886	0.53
13		1.130	0.1563	0.93+
16		1.390	0.0867	0.52
20		1.570	0.0450	0.26
26		1.680	0.0183	
30		1.700	0.0036	
32		1.700	0	

One-half of a 9-day-old culture was filtered through a Berkefeld filter, and the formol titration showed that gelatin decomposition of the filtrate continued in a straight line at the rate of 0.172 mgm. per cubic centimeter in 5 days while the unfiltered culture produced 0.385 mgm. in the same time (table 2). About one-half of the enzyme producing NH_2 and NH_3 groups had passed the filter. The other half may have been inside the cells, or may have been adsorbed by the filter.

The protein-sparing action of glucose

Addition of 0.5 per cent of glucose prevented gelatin decomposition almost completely. In 40 days, the increase in amino nitrogen per cubic centimeter was 1.43 mgm. in gelatin, 0.04 mgm. in gelatin with 0.5 per cent glucose. Since the enzyme is partly extracellular, glucose as such cannot affect the action of such enzyme, though it might influence its action within the cell, and especially the formation and secretion of more proteolytic enzyme. Neutralization of the acid in the glucose culture, from pH 6.0 to 7.2, did not result in increased proteolysis. This shows that absence of proteolysis was not due to inhibition of the enzyme by acid, but to the fact that no proteolytic enzyme had been produced by the cells.

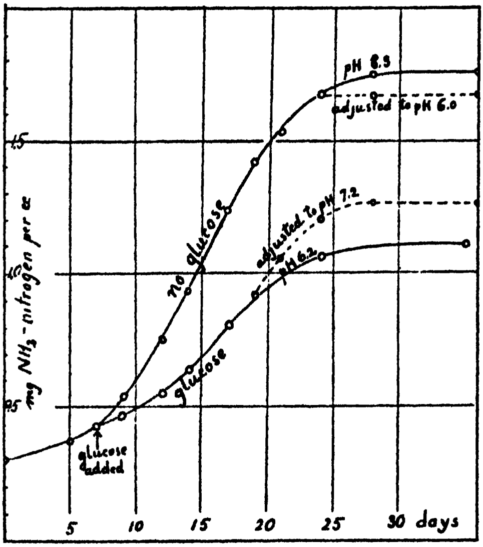
TABLE 2

Milligram $NH_2 + NH_3$ nitrogen in the culture and in its filtrate

AGE OF CULTURE	$NH_2 + NH_3$ NITROGEN PER CUBIC CENTIMETER	
	In culture	In filtrate
days	*mgm.*	*mgm.*
Start	0.308	
4	0.352	
7	0.514	
9*	0.656	0.656
10	0.735	0.700
11		0.726
12	0.864	0.768
14	1.040	0.828

* Day of filtration.

A different picture was obtained when the glucose was added to a 7-day-old gelatin culture (fig. 3). Proteolysis continued in both the original culture and the part to which glucose had been added, though the acid formed from glucose retarded the rate of enzyme action. However, even in this culture, the rate of proteolysis slowly increased as evidenced by the upward trend of the curve. Neutralization from pH 6.2 to 7.2 on the 19th day increased the rate. The culture without glucose reached a pH of 8.3, and when, on the 24th day, this was adjusted to 6.0, proteolysis ceased entirely while it went on a little further in the unchanged part of the culture.



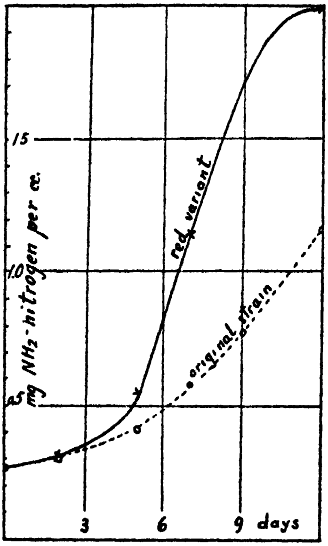


FIG. 3 FIG. 4

FIG. 3. NH_3 FORMATION BY BACILLUS SUBTILIS, WITH AND WITHOUT GLUCOSE ADDITION ON THE 5TH DAY, WITH AND WITHOUT pH ADJUSTMENT

FIG. 4. NH_3 FORMATION BY BACILLUS SUBTILIS AND BY ITS ROUGH, RED VARIANT

While addition of glucose at the start prevents formation of the proteolytic enzyme almost entirely, it does not prevent it when added to a 7-day-old culture. Since the cells in the old culture multiply no more, their internal structure cannot be as readily altered as that of young, growing cells. Aside from the direct effect upon the enzyme formation, we have further an inhibition of proteolysis by the acid formed from glucose.

The need of oxygen for gelatin liquefaction could be established, and the redox-potential seemed to play a rôle, but during the quantitative measurement of these factors, a change of the organism occurred.

Dissociation of the culture

The strain used produces a red pigment on glucose media, but growth is colorless without sugar. The new variant, however, producing a water-soluble red pigment in gelatin without sugar, appeared sometimes after 5 days, sometimes not until the 30th day. Transfers from dissociated cultures produced the pigment in 24 to 48 hours.

The red variant is less dependent upon oxygen than the original, and this prevented accurate measurements. It is Gram-variable. The Gram-positive cells appear granular, and the stained particles look like a streptococcus. Liquefaction is very rapid (see fig. 4). Growth is so agglutinative that plate counts or direct counts become impossible. For this reason, it could not be decided whether the early point of inflection in figure 4 is merely caused by a more rapid growth, or by a change of the character of the enzyme.

This change from the white *B. subtilis* to a red strain has been observed frequently in this laboratory by several bacteriologists. It occurs not only with the laboratory strain mentioned, but also with the Marburg strain.

SUMMARY

Gelatin decomposition by *Bacillus subtilis*, as measured by formol titration, is at least partly due to an extracellular enzyme which passes the Berkefeld filter. Therefore, no definite relation exists between the number of cells and the amount of decomposed gelatin.

Addition of glucose at the start prevents almost entirely the formation of the proteolytic enzyme. Addition to a culture in which multiplication of cells has ceased does not prevent the formation of more enzyme though the rate of enzyme action is decreased by the acid formed from glucose.

Bacillus subtilis dissociates at times to a more rough type with red pigment and more intense action on gelatin.

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THE FERMENTATION OF MUCIC ACID BY SOME INTESTINAL BACTERIA

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Received for publication January 5, 1938

In the course of a study of the fermentation of some sugar derivatives by bacteria, it was found that various intestinal bacteria showed interesting results with mucic acid. *Escherichia coli*, *Shigella dysenteriae*, Sonne-Duval, *Salmonella enteritidis*, and *S. schottmülleri* fermented this compound with the production of acid. *Aerobacter aerogenes* and *Salmonella aertrycke* produced acid and gas. *Eberthella typhi*, *Shigella dysenteriae*, Flexner, *Salmonella cholerae-suis*, and *Salmonella paratyphi* did not ferment the compound (Sternfeld and Saunders, 1937). Inasmuch as the differentiation of these organisms is of interest it was decided to extend the original observation by testing various strains of each organism.

We are deeply indebted to Dr. P. R. Edwards of the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Dr. S. A. Koser of the Department of Bacteriology, University of Chicago, Miss Virginia Breaks of the Children's Memorial Hospital, Chicago, and Mr. Alfred Noyes of the Coroner's Office, Chicago, for supplying us with the various strains of bacteria used in this study.

Mucic acid, the dicarboxylic acid of galactose, was prepared by the usual method from lactose, the sugar being hydrolyzed and oxidized by treatment with concentrated nitric acid. On cooling, the water-insoluble mucic acid precipitates, and after filtration is recrystallized several times from 50 per cent ethyl alcohol. The pure product is obtained in the form of white crystals, melting at 212° and optically inactive.

For fermentation tests, the acid was neutralized with sodium hydroxide and added in 0.5 per cent concentration to ordinary nutrient broth, pH 7.0 to 7.2. Brom-cresol-purple (pH range 5.2 to 6.8) was added as indicator and the medium tubed with inner inverted fermentation vials. Sterilization was carried out by autoclaving, using fifteen pounds of steam for ten minutes. It had previously been found that sterilization of the sugar acid by filtration through a Seitz filter yielded results that in no way differed from those obtained by using the ordinary sterilizing procedure. An incubation temperature of 37°C. was employed in all the tests. The controls in each experiment consisted of a non-inoculated tube of medium. In some experiments a control was used consisting of tubes of ordinary nutrient broth (without the sugar acid) inoculated with each organism. In this fashion, acid production due to a spontaneous breakdown of the sugar or due to abnormal bacterial breakdown of the constituents of the nutrient broth could be detected.

TABLE 1

Fermentation of mucic acid

ORGANISM	ACID	ACID AND GAS	NEGATIVE
A. aerogenes (5)	4	1	0
E. coli (5)	3	1	1
E. typhi (9)	0	0	9
S. paratyphi (4)	0	0	4
S. Schottmülleri (4)	3	1	0
S. aertrycke (21)	9	12	0
S. cholerae-suis (7)	0	0	7
S. enteritidis (12)	3	9	0
S. suipestifer (4)	0	0	4
S. dysenteriae, Shiga (2)	0	0	2
S. dysenteriae, Flexner (4)	0	0	4
S. dysenteriae, Sonne-Duval (8)	6	0	2

Readings were taken once a day for five successive days and then every third day for a total period of two to three weeks. In table 1 are presented the results of the fermentation of mucic acid.

All positive fermentations except two strains of *Shigella* *dysenteriae*, Sonne-Duval, occurred within forty-eight hours, many fermenting within twenty-four hours. The two exceptions showed positive fermentation after nine days. An interesting observation was that most of the strains exhibited an alkaline reaction from forty-eight to seventy-two hours after they had shown a positive fermentation, indicating in all probability a further utilization of the intermediary acid products resulting from the breakdown of the mucic acid.

It is of some interest to compare the results obtained by the use of the mucic acid with those obtained when *d*-tartaric acid was used in fermentation tests for differentiation of some types within the *Salmonella* group (Jordan and Harmon, 1928). *S. paratyphi and S. schottmülleri* did not ferment the tartrate, whereas *S. aertrycke*, *S. enteritidis*, *S. cholerae-suis*, *S. abortivo-equinus*, and *S. herschfeldii* produced acid. Koser (1923) showed that *E. coli* and *A. aerogenes* would, in most cases, grow on a synthetic medium where the only source of carbon was mucic acid. Kendall and Gross (1930) reported that mucic acid was not fermented by *B. paratyphosus*.

Barker (1936) showed that *A. aerogenes* would ferment *d*-tartaric, *l*-malic, and fumaric acids but not *d*-malic, maleic, or succinic acids.

SUMMARY

The fermentation of mucic acid by various intestinal bacteria was tested, using eighty-two strains. With the exception of three strains, the results were consistent in that all the strains of one species either fermented or did not ferment this compound. *Aerobacter aerogenes*, *Escherichia coli*, *Salmonella schottmülleri*, *Salmonella aertrycke*, *Salmonella enteritidis*, and *Shigella dysenteriae*, Sonne-Duval, ferment mucic acid; whereas, *Eberthella typhi*, *Salmonella paratyphi*, *Salmonella cholerae-suis*, *Salmonella suipestifer*, *Shigella dysenteriae*, Shiga and *Shigella dysenteriae*, Flexner, do not ferment mucic acid. Inasmuch as mucic acid is very readily obtained, we believe that this compound will be of help to the bacteriologist working with intestinal bacteria.

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GELATIN AS A SOURCE OF GROWTH-PROMOTING SUBSTANCES FOR BACTERIA[1]

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Received for publication January 29, 1938

In the course of studies on the nutritional requirements of some of the more exacting bacteria it was found that the addition of certain brands of gelatin to a simplified synthetic medium caused growth of a number of bacteria which were unable to develop in the same medium without the gelatin. The use of gelatin in amounts ordinarily employed for culture media produced results which were comparable to, or at times better than, those secured by the use of small amounts of fractionated extracts of spleen, liver or yeast.

Extracts of spleen, liver, yeast and other materials have been subjected by a number of workers to chemical fractionation in attempts to obtain growth-promoting substances responsible for development of the more fastidious bacteria. Since gelatin appeared to offer a promising source of these substances a further study of its effect was made. A comparison of results secured with the use of Difco gelatin and with a typical tissue preparation is given in table 1.

The synthetic medium used in this experiment was similar in composition to that employed in previous work (Koser and Saunders, 1935) and contained inorganic salts, glucose, asparagin, cystine and tryptophane. None of the microorganisms were able to develop in the synthetic medium alone. The amounts of added spleen preparation and of gelatin necessary to secure development of five representative organisms are shown in the

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[1] This investigation was aided by a grant to the University of Chicago from the Rockefeller Foundation.

TABLE 1

Comparative growth-promoting effect of a spleen preparation and of gelatin when added to a synthetic medium

	SPLEEN PREPARATION*				GELATIN (DIFCO)			
	mgm. per cc. added	Development of culture			mgm. per cc. added	Development of culture		
		days				days		
		1	2	4		1	2	4
Staphylococcus albus	None	0	0	0	None	0	0	0
	0.1	+	+++	+++	25.0	++	++++	++++
	0.03	0	+	++	10.0	0	+++	+++
	0.01	0	+?	+	3.0	0	++	+++
					1.0	0	+	++
Streptococcus pyogenes (from scarlet fever)	None	0	0	0	None	0	0	0
	1.0†	+	++	++	50.0	+++	++++	++++
	0.1†	0	0	0	25.0	0	+	++
					10.0	0	0	0
Brucella abortus	None	0	0	0	None	0	0	0
	0.3	+?	++	+++	50.0	+	++	++++
	0.03	0	?	+	25.0	0	+	++
					10.0	0	0	+
Corynebacterium diphtheriae	None	0	0	0	None	0	0	0
	0.1	+	++	+++	25.0	+	+++	+++
	0.01	0	+	++	10.0	+	++	+++
	0.001	0	0	+	1.0	0	+?	++
Shigella paradysenteriae	None	0	0	0	None	0	0	0
	0.1	++	+++	+++	25.0	+++	+++	+++
	0.01	0	+	+	10.0	+	+	+
					3.0	0	0	+

* The spleen preparation had been subjected to a process of fractionation to remove a part of the inert material while preserving the growth-promoting substances.

† The results shown for the streptococcus were secured with crude spleen extract. When similar amounts of partially purified spleen preparation were used there was no growth. Evidently one or more essential substances for this organism are lost in our present method of preparation.

In this experiment the gelatin was autoclaved separately and added to the synthetic medium in the amounts specified.

0 = no visible growth; ? or +? = questionable or very light growth; + to ++++ = light to luxuriant growth.

table. It will be seen that considerably larger quantities of gelatin were required to produce a growth-promoting effect comparable to that resulting from the use of small amounts of spleen preparation. Usually from one hundred to three hundred times as much was needed for this purpose.

A number of other microorganisms, in addition to those listed in the table, were also tested in the gelatin synthetic-medium combination. Among these were four strains of hemolytic streptococci, six of green-producing streptococci from various sources, four strains of meningococci, representatives of types I, II and III pneumococci, four strains of the gonococcus, six strains of *Leuconostoc*, five of *Staphylococcus aureus*, one each of *Brucella abortus* and *Brucella suis*, and the yeast *Saccharomyces cerevisiae*. When 5 per cent Difco gelatin was added to the synthetic medium all of the above mentioned organisms developed. In many cases growth was rapid and a pronounced turbidity was produced within 24 hours at 37°C. In a few cases, however, growth in the gelatin medium was somewhat retarded and scantier in amount. Thus, one of the four strains of meningococcus, a strain of type III pneumococcus, two of the four cultures of the gonococcus and one strain each of *Streptococcus viridans* and *Leuconostoc* gave only a light growth which did not appear until the second to fourth day of incubation.

In the synthetic medium without the gelatin none of the foregoing organisms were able to develop. Transfer of a loopful of synthetic medium to veal-infusion agar slants, blood agar or other appropriate medium at various intervals after inoculation showed that the number of cells gradually decreased until none could be found. This was in decided contrast to the growth in the gelatin tubes. Additional data, showing plate counts of cultures in the gelatin synthetic-medium combination and in the synthetic medium alone, are given in table 2.

Two other organisms were used which, in contrast to the foregoing strains, were able to multiply in the synthetic medium. One of these, *Sarcina lutea*, usually produced marked growth after several days of incubation while the other, *S. paradysenteriae*, Duval-Sonne type, never produced more than a light turbidity in the synthetic medium. These two organisms exhibited a decided contrast to each other with respect to cell multiplication upon the addition of gelatin. Development of the Duval-Sonne dysentery organisms was accelerated by the addition of gelatin. The development of *Sarcina lutea*, however, was markedly retarded by amounts of gelatin above two to three per cent.

TABLE 2

Comparative numbers as determined by plate counts in synthetic medium with and without 5 per cent gelatin

ORGANISMS USED	TIME INTERVAL	PLATE COUNTS	
		Synthetic medium + 5 per cent gelatin	Synthetic medium without gelatin
	hours	*numbers per cc.*	*numbers per cc.*
Diplococcus pneumoniae (Type I)	0	12,000	12,000
	24	150,000	250
	48	4,200,000	150
Streptococcus pyogenes (scarlet fever)	0	8,500	10,500
	24	55,000	100
	48	2,850,000	75
Streptococcus fecalis	0	9,000	8,000
	24	8,000,000	24,000
	48	200,000,000	2,500
Staphylococcus albus	0	10,000	12,000
	24	200,000,000	4,500
	48	120,000,000	2,400
Corynebacterium diphtheriae	0	300,000	270,000
	24	10,000,000	30,000
	48	50,000,000	2,500
Sarcina lutea	0	4,500	5,000
	24	1,000	110,000
	48	15,000	2,500,000
	96	750,000	1,100,000

Difco gelatin was used for these experiments.

Since many of these organisms occur in chains or clusters, the actual cell population is distinctly greater than the figures given by plate counts.

In further work with the whole series of cultures, the effect of varying amounts of Difco gelatin was tried. It was found that the most pronounced growth-promoting effect was usually secured with 5.0 per cent gelatin. With progressively decreasing quantities slower and scantier development occurred. Certain types, as might be expected, were affected to a greater extent than others when the concentration of gelatin was lowered. In general the hemolytic streptococci, pneumococci, *Brucella* and *Pasteurella* required larger amounts than others and either refused to grow or developed poorly when 1 per cent gelatin was used. With 0.1 per cent gelatin only a few organisms were able to multiply sufficiently to produce a visible turbidity and they developed slowly. In this category were several staphylococci and a strain of the diphtheria bacillus. Concentrations of gelatin over 5 per cent seemed to offer no added advantage and in certain instances 10 per cent and 15 per cent concentrations appeared to be somewhat inhibitory.

In some experiments the gelatin was sterilized separately in the autoclave and added to the medium aseptically, while in others the gelatin was dissolved in the synthetic medium and sterilized with it. While there was no very marked difference in the results secured with these two methods, there was an indication in several different experiments that visible growth appeared a trifle sooner and occurred in slightly lower concentrations of gelatin in those cases where the gelatin was autoclaved in the finished medium.

Comparative tests were made with several different brands of gelatin. A sample of Eastman's photographic gelatin and one of ordinary household gelatin were compared with the Difco product. In this connection it should be mentioned that many gelatins are quite acid and require neutralization when used in culture media. Of these several gelatins, that used for photographic purposes represented the purest product and it is interesting that its growth-promoting effect was decidedly inferior to that exhibited by the two other samples. When the photographic gelatin was used only a few of the microorganisms developed to the point of visible turbidity and they were the ones which were able to grow when only small amounts (0.1 to 0.3 per cent) of the Difco product were supplied. Interestingly, the development of *Sarcina lutea* was not restrained by the addition of 3 per cent photographic gelatin, whereas it was markedly delayed by similar amounts of the household or Difco products.

Using an optimum amount of Difco gelatin, a number of variations in the composition of the basic synthetic medium were tried. Since the original medium contained only a limited number of amino acids, another more elaborate synthetic medium was substituted for it. The latter contained small quantities of 15 amino acids, in addition to 0.2 per cent glucose and inorganic salts (Koser, Finkle, Dorfman, Gordon and Saunders, 1938). However, the resultant growth after the addition of Difco gelatin was as a rule no more rapid nor luxuriant than with the restricted synthetic medium.

In other experiments certain of the constituents of the synthetic medium were omitted. Omission of the amino acids resulted in little decrease in either the rapidity or luxuriance of development. Apparently the necessary amino acids, as well as other essential substances, were present in the gelatin preparation (Difco). Omission of both the glucose and the amino acids lowered somewhat the nutritive value of the mixture, particularly when smaller amounts of gelatin (0.3 per cent and 1.0 per cent) were used. Omission of the inorganic salts of the synthetic medium produced a culture environment distinctly inferior to that of the complete medium. A solution of gelatin alone permitted growth of only a limited number of the organisms.

On ashing 5-gram samples of both Difco and household gelatin it was found that the factors responsible for growth were destroyed. The ash which remained after ignition, when added to several different synthetic media in appropriate amounts, exerted no growth-promoting effect whatever. There was no detectable ash after ignition of the Eastman gelatin.

The most obvious explanation of the growth-promoting effect shown by several of the brands of gelatin is that essential organic substances of unknown nature are supplied. Since the organisms refused to develop in a synthetic medium containing a wide assortment of amino acids it may be assumed that the substances responsible for growth are other than the usual amino acids. It is realized, however, that other explanations should be considered. Certain inorganic salts, in addition to those supplied in the synthetic medium, may be present in the gelatin and may be required in addition to organic compounds. The effect, too, might be interpreted as being due to combination of the gelatin with inhibitory amounts of metallic impurities in the medium, or to an alteration in the viscosity or the surface tension of the culture solution from a less to a more favorable level. Since the gelatin used for photographic purposes is supposedly a purer product than other gelatins, one is led to assume that the substances needed by the organisms are present as impurities. It must not be overlooked, however, that some brands of gelatin may contain substances which restrain development and such an effect might lead to the conclusion that growth factors were absent.

It seemed of interest to compare the viscosity of several gelatins which gave quite different results in the tests of growth-promoting activity. To measure viscosity of the gelatin-synthetic medium combination, one cubic centimeter was allowed to flow through a bulb and capillary arrangement and the time was noted. All measurements were made at 40°C. Distilled water was used as the standard. The synthetic medium without gelatin gave results similar to those secured with distilled water. The following readings are typical:

Distilled water	1 minute	5.8 seconds
5.0 per cent photographic gelatin in synthetic medium	6 minutes	12.7 seconds
5.0 per cent Difco gelatin in synthetic medium	6 minutes	11.6 seconds

It is evident that the photographic gelatin and the Difco product gave similar viscosity readings, though the growth-promoting effects were quite different when a 5.0 per cent concentration was incorporated in the synthetic media.

If the effect of the gelatins may be attributed to the presence of certain organic impurities, the nature of these substances becomes a matter of immediate importance since such exacting types as hemolytic streptococci from scarlet fever and pneumococci were able to develop. Recent work on the growth requirements of microorganisms has thrown some light on the needs of several types, especially with respect to substances other than the ordinary amino acids. From the work of Schopfer (1935), Tatum, Wood and Peterson (1936), Williams and Rohrman (1936), Miller (1936), Knight (1937), Mueller (1937), Mueller and Cohen (1937), and Koser, Finkle, Dorfman, Gordon and Saunders (1938) it is evident that vitamine B_1, beta alanine, and nicotinic acid, either singly or in certain combinations, are needed for the development of propionic acid bacteria, staphylococci, the diphtheria bacillus, several strains of yeast and a mold.

Whether the foregoing substances are present in the brands of gelatin exerting a growth-promoting effect has not been determined. However, since a number of the organisms used in our gelatin experiments refused to develop in synthetic media even after the addition of the three foregoing substances, it would appear that other compounds yet to be identified are necessary. This was true especially of the hemolytic streptococci, pneumococci and allied types. The several brands of commercial gelatin constitute a promising source of material for further studies on the extraction and purification of substances needed by these organisms. That such growth-promoting substances seem to be present in several gelatins in considerably smaller quantities than in tissue infusions is not necessarily a disadvantage, since it might be possible to separate them more readily from gelatin than from such sources as liver, spleen and yeast. Further work alone these lines is now in progress.

SUMMARY

Some brands of gelatin exert a growth-promoting effect upon a number of the more exacting bacteria. This effect appears to be similar to that caused by fractions of spleen, liver and yeast and may be demonstrated by the same method. Considerably larger quantities of gelatin, however, are needed to produce an equivalent effect upon culture development.

A purified gelatin, prepared for photographic purposes, showed relatively little growth-promoting effect.

Certain commercial brands of gelatin appear to constitute a promising source of substances essential for the growth of organisms such as streptococci, pneumococci, dysentery bacilli, *Brucella* and other types which refuse to develop in the usual synthetic media.

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INFLUENCE OF pH ON THE DISSIMILATION OF GLUCOSE BY AEROBACTER INDOLOGENES[1]

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Received for publication January 31, 1938

Osburn, Brown and Werkman (1937) observed a critical pH level in the dissimilation of glucose by *Clostridium butylicum* near 6.3. Above this level butyl alcohol and isopropyl alcohol were not formed. The significance of this critical pH was attributed to the fact that in mixtures of acetic (or butyric) acid and its sodium salt below 6.3 there will be "free" acid, i.e., acid which can be distilled from solution and can be converted into alcohols by *C. butylicum.*

This concept of critical pH is applied to the dissimilation of glucose by *Aerobacter indologenes* in the present investigation, particularly with reference to the findings of Reynolds and Werkman (1937), that when sufficient acetic acid is present in a glucose dissimilation by *A. indologenes,* no gaseous hydrogen is evolved and the acetic acid disappearing in the medium is accounted for as 2,3-butylene glycol + acetylmethylcarbinol. Their conclusions were to the effect that acetic acid occurring in the fermentation was transformed into 2,3-butylene glycol + acetylmethylcarbinol.

The principle of critical pH probably has a general application to cellular metabolism. Although pH 6.3 was found to be the critical level in the case of *C. butylicum* in converting butyric and acetic acids into corresponding alcohols, it is likely that each organism will show several such levels which will be determined by the dissociation of the substance subject to change. Furthermore, as pointed out by Osburn and Werkman (1937), the change in metabolism does not occur sharply at pH 6.3, but the effect is gradual and extends over a relatively narrow range.

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[1] Supported in part by Industrial Science Research Funds of Iowa State College.

The dissimilation of glucose by *A. indologenes* at pH levels below and above 6.3 has been studied in the present work. The results are of particular significance for an understanding of bacterial dissimilation as well as having certain practical implications in procedures involving the presence of acetylmethylcarbinol. In the latter respect, reference may be made to the Voges-Proskauer test and the formation of acetylmethylcarbinol in butter cultures.

In the work of Reynolds and Werkman (1937) no mention of a critical pH level at which the mechanism of dissimilation changes its nature, is made. In the present investigation the significance of levels above and below pH 6.3 is shown in the dissimilation of glucose by *A. indologenes*. The results indicate that the gas ratio of carbon dioxide to hydrogen, the occurrence of acetylmethylcarbinol and 2,3-butylene glycol and the amounts of acetic and formic acids are dependent on the pH of the fermentation and that the range near pH 6.3 is a critical level; below this range, the course of dissimilation is markedly different from that above.

METHODS

Glucose plus acetate fermentations were conducted in 4-liter Erlenmeyer flasks fitted with proper openings for removal of gas, taking of samples, and addition of alkali. Brom-thymol-blue was used as an indicator and the pH maintained by adding normal sodium hydroxide.

The medium consisted of glucose 2 per cent, ammonium sulfate 0.3 per cent, peptone 0.5 per cent, sodium acetate 0.6 per cent and 0.1 M dipotassium phosphate. It is of interest that distilled water was not suitable for making up the medium. Tap water provided excellent growth. When Speakman's salts in distilled water replaced the tap water, growth was not nearly as good. When tap water was evaporated and the residue ashed, addition of the latter provided growth equal to that in the tap water medium. Apparently the essential constituent is inorganic.

The pH was adjusted with sulfuric acid and the medium sterilized at 20 pounds pressure for 30 minutes. Glucose was sterilized separately. Incubation was at 30°C.

Carbon dioxide was collected in Bowen potash bulbs in a drying train. Residual carbon dioxide was determined on a sample collected in alkali which was acidified and refluxed in a stream of CO_2-free air. The carbon dioxide was collected and weighed in a Bowen bulb.

Hydrogen was determined by continuous combustion over heated copper oxide and weighing the water formed. Oxygen-free nitrogen was continuously led through the flasks to remove gases.

An aliquot part of the fermented medium was made acid to congo red and distilled to half volume. The distillate was neutralized to phenolphthalein, again distilled and the alcohol determined on this distillate. The two residues were combined and steam distilled to recover the volatile acids. Total volatile acidity was determined on this distillate by titration. The formic acid was determined according to Auerbach and Zeglin (1922), and the acetic acid obtained by difference. In the presence of acetylmethylcarbinol it was necessary to neutralize the volatile acid distillate, evaporate to small volume and remove at least six volumes by alkaline steam distillation to avoid interference with the determination of formic acid.

Ethyl alcohol was determined on an aliquot part of the neutral volatile fraction by oxidation with a sulphuric acid-potassium dichromate mixture in a closed flask on the steam bath. The mixture was then steam distilled and the acetic acid determined. The alcohol determination was corrected for the acetic acid originating from the acetylmethylcarbinol.

Acetylmethylcarbinol was determined on an aliquot part of the fermented medium according to Stahly and Werkman (1935).

2,3-Butylene glycol was steam distilled from a sample of the medium from which the sugar had been removed by the copper-lime technique of Hewitt (1931); 13 volumes or more were collected from a constant volume of 20 ml. which contained 25 grams of $MgSO_4 \cdot 7H_2O$. Butylene glycol was determined on an aliquot part of the distillate by oxidation with periodic acid and directly distilling the acetaldehyde into a solution of sodium bisulphite. The aldehyde was determined by titration with iodine.

The lactic acid was determined on the residue according to Friedemann and Graesser (1933).

The experiments with cell suspensions were conducted in Erlenmeyer flasks containing 300 ml. of 0.1 M phosphate with substrate, adjusted to the desired pH. Carbon dioxide and hydrogen were collected in bottles containing alkali; the former was absorbed and determined on an aliquot part, the latter was determined by volume displacement. The hydrogen evolved was in contact with the medium and was available for reduction of the acetic acid. The inoculum consisted of about 5 grams of cell paste per 300 ml. of medium. Air was displaced from the flasks with nitrogen when the fermentations were started. After four days at 30°C. the flasks were removed and contents analyzed according to the procedure used in the glucose ferementations.

EXPERIMENTAL

In tables 1 and 2 are given results of typical experiments in which the influence of pH on the mechanism of dissimilation of glucose by *A. indologenes* is shown. In the fermentation kept above pH 6.3 (near 7.0) the yields of carbon dioxide and 2,3-butylene glycol are greatly suppressed as compared with an acid fermentation. Acetic and formic acids accumulate and added acetic acid is not attacked. The quantitative relationships of the products are quite different from those in a dissimilation occurring below pH 6.3. Here, acetic acid, even that added, disappears rapidly with the simultaneous occurrence of the 4-carbon compounds, acetylmethylcarbinol and 2,3-butylene glycol (fig. 1); also the production of carbon dioxide is large. Whether the failure of acetic acid to act as an intermediate in alkaline fermentation is due only to a lack of free acid or a dearth of available hydrogen or both, is not clear. Should the presence of formic acid be the result of a synthesis from carbon dioxide and hydrogen, the failure of acetic acid reduction probably results from the utilization of hydrogen in the reduction of carbon

TABLE 1

Fermentation of glucose plus added acetic acid by Aerobacter indologenes. pH maintained above 6.3

TIME	SUGAR FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYLCARBINOL	2,3-BUTYLENEGLYCOL	ETHYL ALCOHOL	2,3-BUTYLENEGLYCOL + ACETYLMETHYLCARBINOL	CARBON RECOVERY	O/R INDEX
hours											*per cent*	
0	0				25.77							
16	19.91	15.03		19.15	37.65	2.72	0.24	9.35	15.20	9.59	108	0.89
20	50.90	33.90		49.90	47.70	2.76	1.27	12.50	36.70	13.77	89	1.06
23½	89.30	51.00		74.00	96.05	7.10	1.50	15.60	64.60	17.10	92	0.97
31½	113.10	58.5	2.26	95.00	109.00	5.85	0	22.40	74.80	22.40	86.3	0.97
75	113.10	60.24	9.26	95.00	109.10	6.35	0	22.30	75.60	22.30	87	0.95

Products in millimoles per liter.

TABLE 2

Fermentation of glucose plus added acetic acid by Aerobacter indologenes at pH 6.3 or below

TIME	SUGAR FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYLCARBINOL	2,3-BUTYLENEGLYCOL	ETHYL ALCOHOL	2,3-BUTYLENEGLYCOL + ACETYLMETHYLCARBINOL	CARBON RECOVERY	O/R INDEX
hours											*per cent*	
0	0				24.66							
24	35.93	59.59		10.40	11.84		3.64	31.40	20.8	35.04	99	0.92
29	48.73	74.57		10.20	7.00	4.76	4.30	42.00	27.16	46.30	102	0.89
33	59.13	86.52		10.08	4.80	2.28	4.64	45.50	31.46	50.14	95	0.88
48	96.98	132.69	1.24	19.72	5.31	2.20	2.20	70.00	51.54	72.20	90	0.87
96	111.3	215.66	17.69	12.20	3.35	2.46	1.05	82.00	58.40	83.05	96	1.12
168	111.3	225.66	29.07	1.35	4.95	1.78	2.36	82.20	55.80	84.56	96.5	1.15
264	111.3	230.74	30.83	1.26	6.00	2.70	0.72	87.20	60.02	87.92	100	1.14

Products in millimoles per liter.

dioxide rather than acetic acid. It is to be noted that there was some carbon dioxide and glycol formed in the alkaline fermentation, probably owing to the difficulty of holding the medium alkaline as at times it became sufficiently acid to allow the formation of these products. In subsequent experiments conducted at pH 7.0 or above, the formation of 2,3-butylene glycol was diminished to a still greater extent—from a normal of approximately 70 mM to 2 mM or less. The accumulation of acetic acid demonstrates its necessity in an available form for the formation of acetylmethylcarbinol and 2,3-butylene glycol. Incomplete carbon recoveries with the alkaline fermentations suggest that some undetermined product is formed.

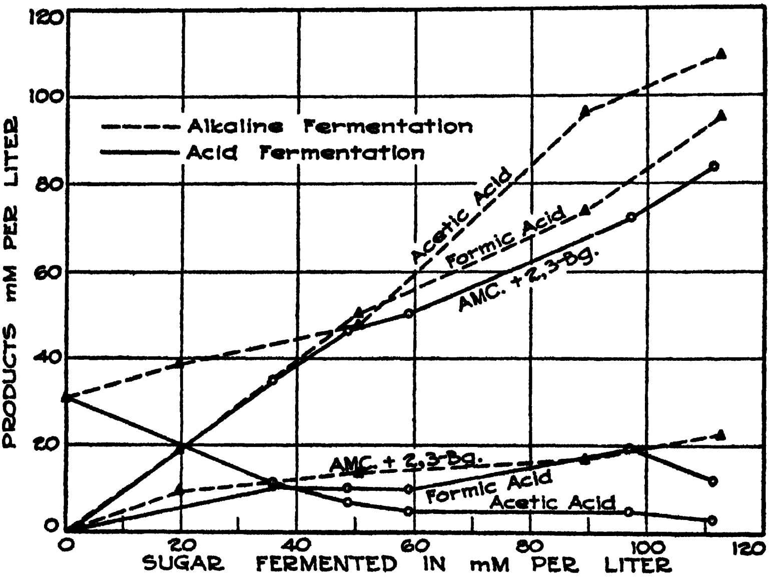

Fig. 1.

It is interesting to note the influence of pH on the gas ratio which varies within large limits. When the pH is maintained below 6.3 and sufficient acetate added, the production of hydrogen is completely suppressed and the carbon dioxide formation is large, whereas, in an alkaline medium the production of both carbon dioxide and hydrogen can be practically prevented. Under the usual conditions of glucose dissimilation the pH drops within the vicinity of 6.0–5.5 but insufficient acetic acid is present to prevent the liberation of hydrogen and a $H_2:CO_2$ ratio of 0.3–0.5 is obtained.

As the fermentation nears completion, the pH shows a reversion owing to the conversion of the acetate ion into neutral compounds.

In regard to the source of hydrogen used in the reduction of acetic acid, it is possible that this is furnished by the dehydrogenation of formic acid. However, using heavy cell suspensions, it was impossible to demonstrate the conversion of acetic acid to acetylmethylcarbinol or 2,3-butylene glycol in the presence of formic acid as H_2-donator (table 3). The fermentations were conducted at pH levels of substantially 6.0–6.2 and at 7.0. It will be observed that the acetic acid was recovered quantitatively in each case and that the formic acid broken down was accounted for quantitatively by equimolar amounts of carbon dioxide and hydrogen. It is also of significance that the formic acid decomposed was about four times greater in an acid than alkaline medium. This finding supports in part, the results from a glucose fermentation under the same conditions. The hydrogen from formic acid did not reduce acetic acid under these conditions. However, in the presence of glucose plus acetic acid the yield of hydrogen is greatly suppressed. If formic acid is the only hydrogen-yielding intermediate it is difficult to account for this behavior which suggests that hydrogen from some other source

TABLE 3

Fermentation of acetic and formic acids by cell suspensions of Aerobacter indologenes in acid and alkaline medium

REACTION OF MEDIUM	ACETIC ACID ADDED	FORMIC ACID ADDED	ACETIC ACID	FORMIC ACID	CARBON DIOXIDE	HYDROGEN	FORMIC ACID FERMENTED
pH 6.0–6.3	112.25	137.76	112.35	72.85	62.20	60.56	64.91
pH 7.0–7.3	111.53	136.90	113.30	121.16	12.40	13.38	15.74

Voges-Proskauer test negative. Products in millimoles per liter.

is necessary for the conversion of acetic acid to acetylmethylcarbinol. Formic acid hydrogen is able to reduce acetylmethylcarbinol to 2,3-butylene glycol by *Escherichia coli* (table 4).

The effect of pH on the action of the formic hydrogenlyase is important. Krebs (1937) found that *E. coli* did not form lactic acid from pyruvic acid anaerobically when the pH was between 6.6–8.0. However, if the pH was lowered to 5.4, considerable lactic acid and carbon dioxide were produced. In an alkaline medium formic and acetic acids were the only final products of pyruvic breakdown. Woods (1936) found in the case of *E. coli* that an alkaline reaction is necessary for a synthesis of formic acid from carbon dioxide and hydrogen. We have found that formate in an acid medium is decomposed vigorously. However, at alkaline levels the conditions are relatively more favorable for the synthesis of formic acid than its breakdown. Since formic hydrogenlyase is a reversible system, the retention of carbon dioxide in the medium may tend to drive the reaction toward the synthesis of formate.

TABLE 4

Reduction of acetylmethylcarbinol by cell suspensions of Escherichia coli and formic acid

	ACETYLMETHYLCARBINOL	CARBON DIOXIDE	HYDROGEN	FORMIC ACID	ACETIC ACID	2,3-BUTYLENE-GLYCOL	CARBON RECOVERY
							per cent
Initial	54.25			112.77			
Final	0	76.31	30.10	39.69	25.73	41.91	102

Products in millimoles per liter.

SUMMARY AND CONCLUSIONS

A critical pH level has been shown to exist in the dissimilation of glucose by *A. indologenes* in the region of 6.3. Fermentation carried out above this level results in an accumulation of acetic and formic acids. The production of hydrogen and carbon dioxide is greatly suppressed and the formation of acetylmethylcarbinol and 2,3-butylene glycol may be prevented if care is taken not to allow the pH to drop within the vicinity of 6.3. When the fermentation occurs below pH 6.3, the acetic acid is converted into acetylmethylcarbinol and 2,3-butylene glycol. If sufficient acetic acid is added to an acid fermentation, the production of gaseous hydrogen is prevented.

It is suggested that the acidity and alkalinity of the medium expresses itself by determining the relative hydrogen accepting ability of acetic acid and carbon dioxide. When the medium is alkaline, carbon dioxide is the better hydrogen acceptor resulting in the formation of formic acid; under acid conditions (approximately 6.3 or less) carbon dioxide cannot compete with acetic acid and acetylmethylcarbinol and 2,3-butylene glycol are formed.

For the conversion of acetic acid into neutral compounds, it is important to have both free acetic acid and available hydrogen. Evidence suggests that the general occurrence of formic acid among the final products of bacterial dissimilation of glucose may be the result of a synthesis from carbon dioxide and hydrogen, a fact which would have important implications in formulating schemes of dissimilation particularly with reference to the breakdown of pyruvic acid into formic and acetic acids or acetaldehyde and carbon dioxide.

The results point clearly to the fact that the gas ratio of ($H_2:CO_2$) 0.5 is fortuitous. This generally accepted ratio of 0.5 is the result of the conditions under which the determination is made and may vary within wide limits. Review of the literature dealing with quantitative experiments shows that the ratio is usually near 0.3, indicating a marked utilization of hydrogen for the reduction of acetic acid to 2,3-butylene glycol.

Appreciation is expressed to Dr. A. R. Stanley for assistance in preliminary experiments.

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THE FERMENTATION OF DISACCHARIDES BY STREPTOCOCCUS THERMOPHILUS

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Received for publication February 2, 1938

Orla-Jensen (1919) was the first to differentiate and describe *Streptococcus thermophilus* as a distinct streptococcal species. Among the unique characteristics of this organism which were pointed out by Orla-Jensen are its general inability to ferment maltose, and its ability to ferment sucrose and lactose with especial vigor, the latter disaccharides being even more favorable to the growth of this organism than are the simple monosaccharides. Orla-Jensen also noted the very interesting fact that *Streptococcus thermophilus* does not ferment mannose, or ferments it very feebly.

Wright (1936) has also noted that some strains of *Streptococcus thermophilus* ferment lactose and sucrose more rapidly than they do the constituent monosaccharides, and on the basis of these results he suggests that *Streptococcus thermophilus* ferments the disaccharides without preliminary hydrolysis. In times past it was a common belief that the lactic-acid bacteria caused a direct fermentation of the disaccharides, since the presence of monosaccharides cannot usually be demonstrated in fermenting cultures by chemical means; even then, however, most investigators held to the theory that the disaccharides were hydrolyzed to the constituent monosaccharides as the first step in their fermentation.

Some years ago one of us became interested in this problem in connection with *Streptococcus lactis*. It was thought that although the preliminary hydrolysis of the disaccharides was not easily proved by chemical means, an indication that such hydrolysis actually occurs might be demonstrated by the use of bacteria. It was found that if a culture of *Streptococcus lactis* (sucrose +) and a culture of *Bacterium coli* (sucrose −) are grown together in sucrose broth, gas is formed, though neither of these organisms alone can produce gas from sucrose. Also, when *Streptococcus lactis* (lactose +) and *Proteus* (lactose −) are grown together in lactose broth, gas is likewise produced. At that time it did not appear that this fact had been demonstrated with *Streptococcus lactis.* However, our results were not published, because a survey of the literature showed that such phenomena had been encountered in connection with "false presumptive tests" for *Bacterium coli* in water, in which cases the gas produced from lactose appeared to result from the cooperative action of such organisms as *Streptococcus fecalis*, which can ferment lactose without gas production, and other bacteria capable of producing gas from glucose though not attacking lactose. These facts naturally suggested themselves in connection with Wright's idea concerning the direct fermentation of disaccharides by *Streptococcus thermophilus.*

That *Streptococcus thermophilus* appears to utilize sucrose and lactose more readily than the monosaccharides is easily demonstrated by the rate of acid production in media containing these sugars. Table 1 shows the relative rates of fall in pH with two cultures of *Streptococcus thermophilus* in broths containing 1 per cent of the respective sugars together with 0.3 per cent each of beef extract, peptone and tryptone. Galactose, not included in the table, was fermented more slowly than glucose, as Wright has previously shown.

Streptococcus thermophilus and *Bacterium coli* were grown separately and together in sucrose broth. Since the strain of *Bacterium coli* used could not ferment sucrose, neither of these organisms alone is able to produce gas from this sugar whereas gas production resulted when the two organisms were grown together. The results obtained are shown in table 2.

Experiments were also conducted with *Streptococcus thermophilus* and *Proteus* (lactose −) in lactose broth. The findings (table 3) are in agreement with those obtained with sucrose.

Tests similar to those reported in tables 2 and 3 were conducted with two strains of *Streptococcus thermophilus* and the experiments were repeated a number of times in media of different composition.

TABLE 1

The rate of fermentation (pH) of hexoses and disaccharides by Streptococcus thermophilus

CULTURE	HOURS	GLUCOSE	FRUCTOSE	SUCROSE	LACTOSE
2	0	6.6	6.6	6.6	6.6
	24	6.4	6.2	5.2	5.3
	66	6.0	4.4	4.7	4.3
	168	5.0	4.4	4.5	4.2
6	0	6.6	6.6	6.6	6.6
	24	6.5	6.2	5.3	5.4
	66	6.0	4.4	4.7	4.3
	168	5.0	4.4	4.5	4.2

TABLE 2

Fermentation of sucrose by Streptococcus thermophilus and Bacterium coli

INOCULUM	ACID	GAS
S. thermophilus	+	–
Bact. coli	–	–
S. thermophilus + *Bact. coli*	+	+

TABLE 3

Fermentation of lactose by Streptococcus thermophilus and Proteus

INOCULUM	ACID	GAS
S. thermophilus	+	–
Proteus	–	–
S. thermophilus + *Proteus*	+	+

The formation of gas was revealed by the use of test tubes of broth with vaseline seals, but in some tests it was also demonstrated with the use of inverted Durham tubes.

To repeat these results, a little attention should be paid to the composition of the medium. In a lightly buffered medium, if *Streptococcus thermophilus* gets a rapid start the associated organisms may be inhibited before visible evidence of gas is obtained. On account of the extreme sensitiveness of *Streptococcus thermophilus* to salt (Sherman, 1937), it may not do well in media heavily buffered with phosphates. We have found a suitable medium to be one containing 1 per cent of the test sugar with 0.3 per cent each of beef extract, peptone, tryptone, and dibasic potassium phosphate, though several other media have been used successfully.

Although the name *Streptococcus thermophilus* has been loosely used by some workers for various heat-tolerant streptococci, this organism has a very peculiar combination of characteristics which widely separates it from the other clearly defined streptococcal types. In our own work covering a considerable experience in addition to our published data (Sherman and Stark, 1931), the various strains studied have made up a remarkably homogeneous group, though somewhat greater variation has been noted by others who have undoubtedly dealt with the true *Streptococcus thermophilus.*

With reference to the fermentation of disaccharides by *Streptococcus thermophilus,* these simple experiments should not be accepted as offering definite proof; they do, however, indicate that this organism probably hydrolyzes the disaccharides to their constituent monosaccharides as the initial step in the fermentation; they do, also, put the burden of proof on those who hold the opposite view.

SUMMARY

It is shown that if *Streptococcus thermophilus* (sucrose +) and *Bacterium coli* (sucrose −) are grown together in sucrose broth, gas is produced, whereas neither of these organisms alone can produce gas from sucrose. Likewise, gas is produced in lactose broth when *Streptococcus thermophilus* (lactose +) and *Proteus* (lactose −) are grown together.

These results suggest, in opposition to one view which has been held in the past, that *Streptococcus thermophilus* probably hy-

drolyzes the disaccharides to their constituent monosaccharides in the fermentation of such sugars.

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INFLUENCE OF TIME AND TEMPERATURE OF INCUBATION ON HEAT RESISTANCE OF ESCHERICHIA COLI[1]

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Received for publication February 3, 1938

Field and laboratory observations (Frazier *et al.* 1935) long have indicated that the temperature of incubation of Swiss cheese starter cultures significantly influences their ability to develop following the rather severe heat exposure to which they are subjected during manufacture. If it were true that the temperature of growth had an effect on heat resistance of bacteria, this fact would be of significance in various fermentations, both commercial and natural, and would prove of general interest from the standpoint of the physiology of bacteria. Therefore, an investigation was initiated to determine the influence of incubation temperature and time on the thermal resistance of certain Swiss cheese starter cultures. For the purpose of comparison, similar studies were undertaken with a typical strain (H-52) of *Escherichia coli*. The results of the investigations on *E. coli* are presented in this paper.

Because it produced less acid, was able to develop under a wider variety of environmental conditions, and could be counted fairly accurately by the plate method, *E. coli* gave results which were more conclusive than those obtained with the lactic starter cultures and indicated that *E. coli*, a favorite subject for experimentation, is an ideal organism for studies on the heat resistance of vegetative cells.

Despite the apparent importance of the relationship between the growth temperature and thermal resistance of bacterial cells,

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[1] This work has been aided by a grant from the Wisconsin Alumni Research Foundation.

few attempts have been made to correlate them. That the growth temperature may directly influence the thermal relationships of protozoa has been demonstrated by Dallinger (1887), who by gradually increasing the incubation temperature of three flagellates over a seven-year period raised their maximum temperature from 23° to 70°C. At the same time the optimum and minimum temperatures were raised to such a degree that when cells growing at 70°C. were placed at 15°C. they perished.

Sherman and Cameron (1933) found that when young cultures of *E. coli*, grown at 45°C., were placed in sterile media at 10°C., there was a far greater destruction of cells than when young cultures grown at 10°C. were placed in sterile media at 45°C. The same investigators reported (1934) that young cells of *E. coli* from cultures growing slowly exhibited greater resistance to various deleterious factors than did the cells from cultures growing rapidly. The growth rate was reduced by using low incubation temperatures, dilute media or media of increased osmotic pressure.

Frazier and coworkers (1935) demonstrated that a slight elevation in incubation temperature from 35–37° to 38–39°C. markedly increased the ability of *Lactobacillus helveticus* to develop at high temperatures.

According to Anderson and Meanwell (1936) a thermoduric streptococcus in the lag and early logarithmic phases of growth showed increased resistance to heat when the incubation temperature was reduced below the optimum.

Dorner and Thöni (1936) found that after cells of *Bacterium acidi-propionici* had reached the mature, heat resistant stage, there was little difference between the thermal resistance of cells grown at 22° and at 30°C.

Claydon (1937) reported that at 10°C. *Streptococcus lactis* cultures grew more slowly than at higher temperatures, but attained greater thermal resistance.

Theophilus (1935) demonstrated that spores formed at the optimum temperature for growth were definitely more heat resistant than those formed at temperatures either below or above the optimum.

The influence of age of bacterial cells on their thermal resistance has been more widely studied.

Numerous investigators including Reichenbach (1911), Schultz and Ritz (1910), Sherman and Albus (1923, 1924), Behrens (1923), Hückel (1926), Ørskov (1925), Darányi (1927), Gates (1929), Jensen (1928), Robertson (1927, 1928), Stark and Stark (1929, a, b), Sherman and Stark (1929), Fabian and Coulter (1930), Hammer and Hussong (1931), Frazier and Wing (1931), Heiberg (1932), Dorner and Thöni (1936), and Claydon (1937) have demonstrated by various methods that young cells are far more susceptible to adverse environmental influences than are older, more mature bacterial cells.

EXPERIMENTAL

In order to maintain the most uniform conditions possible during these investigations, all cultures were incubated in thermostatically controlled water baths, and the time and temperature of incubation were carefully controlled. Moreover, all cultures were carried in freshly prepared, sterile reconstituted skim milk always prepared from the same lot of skim milk powder, and, unless otherwise indicated, one per cent of inoculum was used. Stock cultures, transferred weekly, were incubated at 37°C. for 24 hours after which they were kept at 10°C. until the time for the next transfer.

When it was necessary to inoculate cultures for studies of heat resistance, an inoculating culture was prepared by transfer of one per cent of inoculum from the most recent stock or mother culture to a six-ounce bottle containing 100 cc. of milk. Unless otherwise indicated, this bottle was then incubated under the same conditions as the stock or mother culture and, following incubation, was placed at 10°C. for 36 hours. Then, at varying intervals, inoculations were made from the inoculating culture into triplicate tubes, each containing 10 cc. of milk. The tubes were incubated for the periods and at the temperatures desired. When the triplicate tube cultures were of the required age, they were removed from the water bath. One cubic centimeter of culture from each of the triplicates was placed in a test tube containing 3 cc. of sterile 2 per cent sodium citrate. The contents were thoroughly mixed; 0.1 cc. was transferred to a tube containing 10 cc. of milk at a temperature of 10°C.; and a sample was removed for the plate count. The tubes were then placed in a mechanically stirred water bath at a temperature of 54°C.; one minute was allowed for the temperature rise, and then heat treatment was carried out at 53°C. for 30 minutes, after which treatment the tubes were immediately cooled and samples removed for plate counts. Plain nutrient agar was used, and plates were incubated at 37°C. for 48 hours.

Curran and Evans (1937) have shown that the indicated percentage survival of cells during heat treatment may be greater if some medium is employed which is superior to plain nutrient agar. Addition of a fermentable carbohydrate to the agar was undesirable because of the resulting gas formation. The results obtained with plain nutrient agar were very uniform and it appeared to be most suitable for these particular studies.

When larger samples were desired, one-fourth per cent of the culture to be heat shocked was transferred to Erlenmeyer flasks containing 450 cc. of milk. The temperature of the milk in the flasks was raised to 53°C. in a period of about five minutes, maintained at 53°C. for 30 minutes and then lowered. Plate counts were made before and after heat treatment.

Percentage survival of mature cells grown at various temperatures

In a preliminary experiment, inoculating cultures were prepared from stock cultures, incubated, respectively, at 28°, 30°, 30.5°, 38.5° and 40°C. for varying periods, and then transfers were made from the inoculating cultures to flasks of milk. The inoculated flasks were heated at 53°C. for 30 minutes, and the heat resistance of the respective cultures was determined. Table 1 shows the percentage survival during heat treatment of cultures which had been incubated for varying periods at the six different temperatures.

These results demonstrate that during the maximum stationary phase of growth the maximum percentage survival of cells at 38.5° and 40°C. is distinctly greater than that obtained at lower temperatures such as 28°, 30° and 30.5°C. Heat resistance appears to be lowest at 28°C., the lowest incubation temperature employed.

TABLE 1

Heat resistance of Escherichia coli grown at different temperatures for varying periods and then heat shocked at 53°C. for thirty minutes

TEMPERATURE OF INCUBATION	TIME OF INCUBATION	PLATE COUNT		PERCENTAGE SURVIVAL
		Before heating	After heating	
°C.	*hours*	*nos. per cc.*	*nos. per cc.*	
28	38	1,467,000	120,000	8.2
	42	1,445,000	120,000	8.3
	48	1,540,000	93,000	6.0
	54	1,447,000	113,000	7.8
	60	1,333,000	107,000	8.0
30	37	1,674,000	577,000	34
	42	1,393,000	383,000	28
	46	1,233,000	377,000	31
	51	1,357,000	320,000	24
30.5	42	1,510,000	427,000	28
	48	1,390,000	300,000	22
	54	1,257,000	367,000	29
38.5	21	1,341,000	875,000	65
	24	1,188,000	948,000	79
	27	1,145,000	952,000	83
40	10	1,413,000	267,000	19
	12	1,480,000	533,000	36
	16	1,623,000	753,000	46
	20	1,143,000	827,000	72
	24	1,352,000	770,000	57

Heat resistance of cultures of Escherichia coli carried continuously for two weeks at 28° and 38.5°C., respectively

The results shown in table 2 indicate the heat resistance of cultures of *E. coli* after only one incubation period at various temperatures. However, it was considered possible that the heat resistance of a culture might be altered by numerous successive transfers at a definite temperature. Therefore, mother cultures were inoculated from the stock cultures and carried in tubes containing 10 cc. of milk. According to the results of the previous experiment, shown in table 1, the maximum heat resistance at 28° was maintained from at least the 38th to the 60th hour, and at 38.5° it occurred around the 24th to the 30th hour. In order to make the transfers at the two temperatures at about the time of maximum heat resistance, the 28° culture was transferred every 48 hours and the 38.5° culture every 24 hours. Heat resistance determinations were made after the first transfer and after two weeks of successive transfers at the two temperatures.

TABLE 2

Heat resistance of cultures of Escherichia coli carried at 28° and 38.5°C., respectively, and heat shocked at 53°C. for thirty minutes

INCUBATION OF MOTHER CULTURE		NUMBER OF TRANSFERS	PLATE COUNT		PERCENTAGE SURVIVAL
Temperature	Time		Before heating	After heating	
degrees	*hours*		*nos. per cc.*	*nos. per. cc.*	
28	48	1	1,133,000	253,000	22.3
		1	1,327,000	130,000	9.9
38.5	24	1	1,540,000	960,000	62.3
		1	1,417,000	1,083,000	76.4
28	48	7	1,400,000	250,000	17.9
		7	1,570,000	160,000	10.2
38.5	24	14	1,250,000	1,040,000	83.2
		14	1,490,000	1,210,000	81.2

The cultures to be heated were transferred to flasks of sterile milk and then heat shocked at 53°C. for 30 minutes. Plate counts were made before and after heating. According to the results shown in table 2, the maximum resistance at 28° is again far lower than at 38.5° after both one and numerous transfers at the two temperatures. There seems to be little doubt that when *E. coli* is grown under the conditions of this experiment, thermal resistance during the maximum stationary phase, the time when a culture is considered by most workers to be at the peak of its heat resistance, is far greater when a higher incubation temperature is used. The results further suggested that a slight increase in heat resistance occurred as a result of numerous successive transfers at the higher temperature.

Influence of age of cells and incubation temperature on heat resistance of E. coli

Next a study was made of the progressive changes taking place in the heat resistance of cultures grown at 28° and 38.5°C. during periods varying from the time of inoculation to the end of the maximum stationary phase. Because of the large number of samples to be heat shocked and plated at one time, triplicate tubes were inoculated from the respective inoculating cultures at varying intervals. The inoculating cultures were grown at the temperature to be used in the experiment. The results contained in tables 3 and 4 and figures 1 and 2 reveal certain changes in heat resistance undergone by *E. coli* as it passes through the various growth phases. The heat resistance at the 0 hour is naturally that of the inoculating culture. When active reproduc-

TABLE 3

Heat resistance of cultures of Escherichia coli grown at 28°C. for varying periods and then heat shocked at 53°C. for thirty minutes

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before heating	After heating	
hours	*nos. per cc.*	*nos. per cc.*	
0	25,200	1,600	6.4
1.5	33,800	11,500	34.0
3	40,350	550	1.4
6	297,000	320	0.11
9	730,000	140	0.02
12	1,350,000	540	0.04
15	2,730,000	5,120	0.19
18	3,410,000	58,000	1.7
21	3,000,000	55,000	1.8
24	3,170,000	71,800	2.3
27	3,240,000	83,000	2.6
30	2,710,000	129,000	4.8
33	2,560,000	184,000	7.2
36	3,200,000	184,000	5.8

tion commences, the heat resistance decreases and according to the actual numbers of survivors shown in the table, it would seem that the survivors are cells which had not reproduced in the culture. As the rate of reproduction diminishes, there is a corresponding rise in heat resistance until a peak is attained well along in the maximum stationary phase of growth. There follows a period which varies with the incubation temperature, during which period both the numbers of organisms and the heat resistance remain fairly constant, and after which there is a slow

TABLE 4

Heat resistance of cultures of Escherichia coli grown at 38.5°C. for varying periods and then heat shocked at 53°C. for thirty minutes

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before heating	After heating	
hours	*nos. per cc.*	*nos. per cc.*	
0	25,170	18,500	74
1	32,300	5,450	17
2	78,700	1,000	1.3
3	233,000	6,700	2.9
4	617,000	3,300	0.53
5	990,000	3,300	0.33
6	1,365,000	3,300	0.24
7	2,113,000	3,300	0.15
8	2,540,000	6,700	0.27
9	3,150,000	60,000	1.9
10	2,910,000	53,000	1.8
11	2,820,000	140,000	5.0
12	2,466,000	290,000	12
13	2,680,000	460,000	17
14	2,780,000	540,000	19
15	2,740,000	1,260,000	46
16	2,514,000	1,160,000	46
17	2,114,000	1,380,000	65
18	2,900,000	1,520,000	52
19	2,660,000	2,120,000	80
20	2,320,000	1,340,000	58
21	2,780,000	1,780,000	64
22	2,840,000	1,720,000	61
23	2,880,000	1,993,000	69
24	3,360,000	1,880,000	56

and gradual decline in numbers and in resistance of the cells. Again the heat resistance of the 38.5° culture is far greater than that of the 28° culture. The one and one-half hour sample at 28° indicates an abrupt and brief increase in heat resistance comparable to that reported by Anderson and Meanwell (1936) for a thermoduric streptococcus.

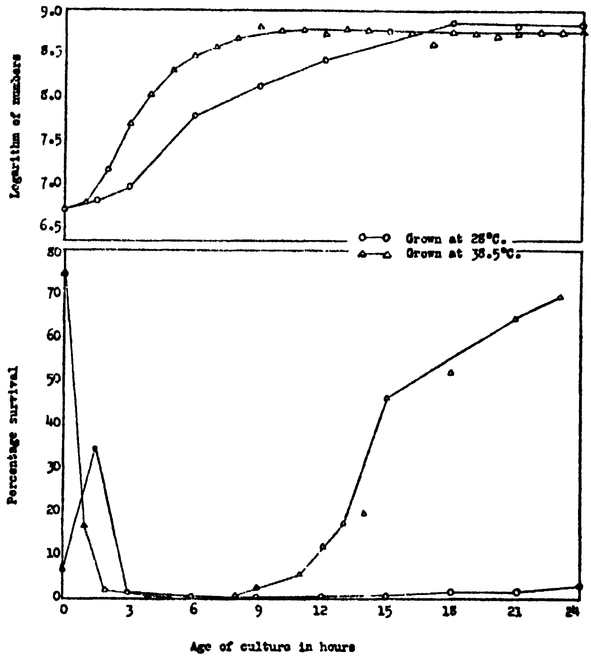

FIG. 1. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival of cells during heat treatment at 53°C. for thirty minutes. Age of cultures, 0 to 24 hours.

Heat resistance of cultures of E. coli during the initial stationary growth phase

The observation that there was an increase in resistance during the early hours of incubation at 28°C. led to a more thorough investigation of the heat resistance of *E. coli* during the first few hours of growth at 28° and at 38.5°C. Inoculating cultures, prepared from stock cultures, were incubated, respectively, at

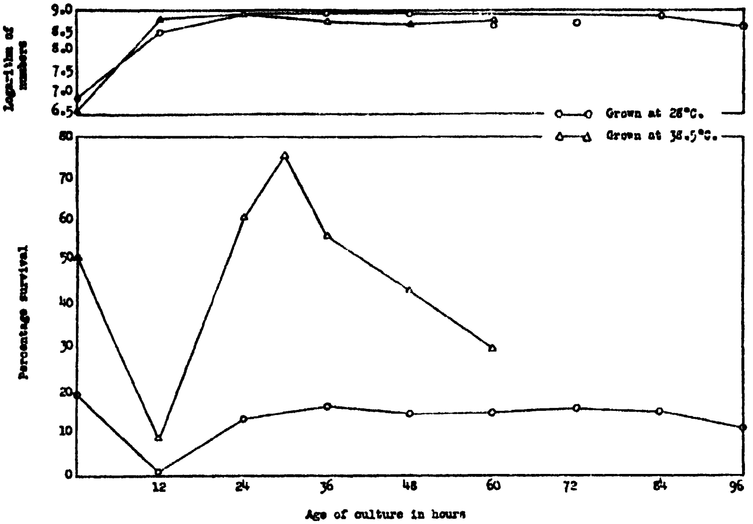

FIG. 2. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival during heat treatment at 53°C. for thirty minutes. Age of cultures, 0 to 96 hours.

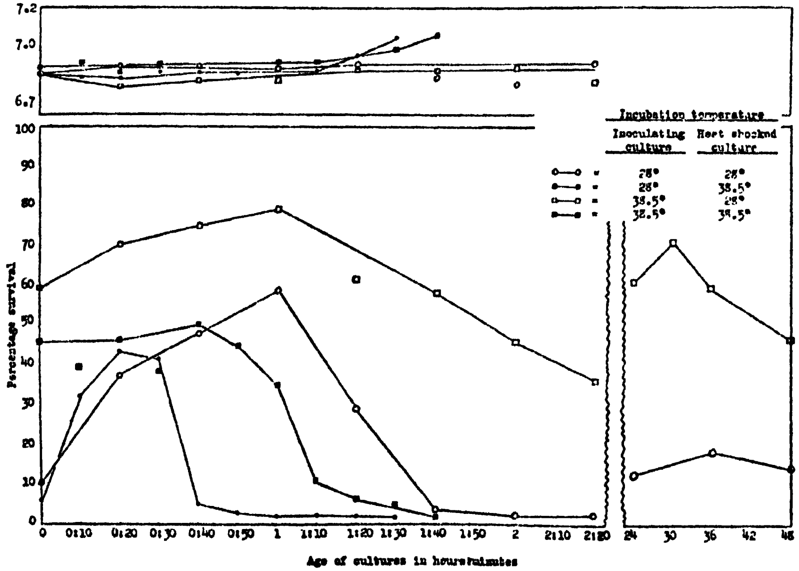

FIG. 3. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival of cells during heat treatment at 53°C. for thirty minutes.

28° for 48 hours and at 38.5°C. for 24 hours. Triplicate tubes were inoculated from these at varying intervals and incubated at 28° and 38.5°C., respectively. To determine the heat resistance of the tube cultures during the first three hours, the percentage survival was calculated. The results are contained in figure 3. The highest percentage survival obtained during the maximum stationary phase of growth at 28° and 38.5°C., respectively, is also shown for purposes of comparison.

Contrary to expectations, it was found that just before or during the "period of physiological youth" there was a distinct, abrupt rise in heat resistance of the culture, and then, a short time before reproduction commenced, a rapid decline in resistance to the low level characteristic of the phase of logarithmic growth.

However, the maximum percentage survival during the initial stationary phase of a culture inoculated from one grown at 38.5°C., generally exceeded that obtained with the subculture from the 28°C. inoculating culture. The greatest thermal resistance was demonstrated by 28° cultures inoculated from a 38.5°C. inoculating culture. When a 38.5°C. inoculating culture was transferred to fresh media with incubation at 38.5°C., no increase in heat resistance occurred during the lag phase of this subculture. These results, therefore, suggest that apparently some factor inherent in the inoculum plays an important rôle in determining the degree of heat resistance during the lag phase of growth.

DISCUSSION

It has been a common belief that a culture of bacteria, in any but the logarithmic growth phase, when placed in a medium advantageous for growth, passed through a stationary or lag phase and underwent a process of "biological rejuvenescence" which was followed by active reproduction. It has been believed that the heat resistance of the culture was notably low during the period of physiological youth and remained low as long as reproduction took place at a rapid rate. It is now apparent that still another change is manifest during the early life of a culture, and that this transitory change is characterized by a very decided increase in heat resistance. This increase is apparently more marked when the culture is placed at a temperature below the optimum for growth.

In these experiments, it was found that during the period of most active reproduction at both 28° and 38.5°C. the heat resistance declined to its lowest point. Then as the cultures entered the maximum stationary phase, their heat resistance again rose. At 28°, however, the maximum percentage survival rarely reached more than 20 per cent and was usually less than 10 per cent, while at 38.5°C., 50 to 80 per cent was usually reached. At 30° the percentage survival was generally about 25 to 30 per cent. The maximum resistance at 40° and 42° was similar to that at 38.5°C. At the higher temperatures, therefore, the maximum percentage survival during the maximum stationary phase in every case exceeded that obtained at the temperatures below the optimum for growth. There are two possible explanations for such an effect of incubation temperature on heat resistance. The temperature at which the organisms are grown may in some manner influence the physico-chemical properties of the cells and thus render them more or less resistant to heat. The fact that the difference between heat resistance of 28° and 38.5° cultures was evident on the first transfer at the two temperatures would tend to discount this theory. Another explanation might be the following: At 38.5°C. reproduction and accompanying changes take place at about twice the rate at 28°C. Therefore, a greater accumulation of mature cells may be possible at 38.5° than could take place at any one time at 28°C. As a result, the maximum percentage survival during the maximum stationary phase would be decidedly greater at the higher incubation temperature.

In addition to their interest from the standpoint of bacterial physiology, the results reported above are of significance for various other reasons. *E. coli* has long been used to determine the efficiency of different methods of destroying vegetative cells. It is apparent that the resistance of *E. coli* to heat and possibly to other adverse factors varies markedly with the time and temperature of incubation and that these, together with the medium in which the organisms are grown before or following heat or other exposure, will play an important part in determining the number of surviving cells.

As indicated by Anderson and Meanwell, organisms other than *E. coli* may increase decidedly in heat resistance during the early hours of growth. Therefore, the temperature at which milk is held previous to pasteurization may profoundly influence the efficiency of the process. The survival of organisms during other processing treatments, intended primarily to decrease the number of vegetative cells in a liquid, may be affected in the same manner.

It has been shown by Elliker (1937) that the culture medium and time and temperature of incubation might have a significant effect on the activity of Swiss cheese starter cultures. Lactobacilli growing more rapidly in a favorable medium generally demonstrated greater heat resistance than did those growing more slowly in a poor medium. Furthermore, when the same organisms were grown in a poor medium, a slight elevation of the incubation temperature and, therefore, a consequent increase in rate of growth usually resulted in a more heat resistant culture. In the present studies with *E. coli*, greatest heat resistance during the maximum stationary phase was exhibited at the higher temperatures where rate of growth had been comparatively rapid.

The results of Anderson and Meanwell and the observations reported in this paper indicate that starter cultures may also exhibit an increase in resistance during the initial stationary phase of growth and that this increased thermal resistance may significantly influence the behavior of the starter organisms during the early stages of the cheese making process, particularly if the cheese be one like Swiss where comparatively high temperatures are employed in the making process.

Future investigations may determine whether or not other species of bacteria demonstrate the same changes in thermal resistance as do the thermoduric streptococcus used by Anderson and Meanwell and *Escherichia coli*.

SUMMARY

Cultures of *Escherichia coli* exhibited a decided increase in heat resistance, as evidenced by percentage survival of cells during heat treatment, while in the initial stationary phase of growth. The increase in heat resistance was more marked in cultures incubated at 28° than in those incubated at 38.5°C. The time and temperature of incubation of the culture used for inoculum decidedly influenced the degree of increase in heat resistance during the initial stationary phase of growth of the subculture.

The heat resistance of all of the cultures decreased as reproduction commenced and their resistance fell to a minimum during the period of most active reproduction. The resistance then increased again to a second peak as the rate of reproduction decreased and the culture entered the maximum stationary phase of growth.

Growth at and above the optimum temperature resulted in cultures whose heat resistance during the maximum stationary phase was distinctly greater than was true of cultures incubated at temperatures below the optimum for growth.

Possible reasons for the variations between heat resistance of cultures grown at high and low temperatures and the practical significance of the results are discussed.

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BOUND WATER CONTENT OF VEGETATIVE AND SPORE FORMS OF BACTERIA

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Received for publication February 5, 1938

In a previous paper (Henry and Friedman, 1937) we confirmed the works of Dyrmont (1886) and Virtanen and Pulkki (1933) which showed that little difference exists in the water content of vegetative cells and spores of a given species of bacteria. These results indicated that the commonly accepted idea that a low water content in the spore form is responsible for the observed heat resistance of this type of cell was not justified.

Virtanen and Pulkki advanced the theory that the enzymes present in the bacterial spore were in an inactive or resistant form. We suggested that the resistance, whether it concerned the enzymes or the bacterial protoplasm proper, might be due to differences in the percentage of bound water in the two types of cells. This suggestion was based on the report of Newton and Martin (1930) which shows that the resistance of certain plants to drought and freezing is, in part, due to their relatively high percentages of bound water.

The present paper is a report of the relative amount of bound water found in the vegetative cells and spores of *Bacillus mycoides*, *Bacillus megatherium* and *Bacillus subtilis*, as determined by the cryoscopic method (Newton and Gortner, 1922). This method was chosen because of its relative simplicity and because of the similarity of our problem to that of Skovholt and Bailey (1935) when they determined the bound water in flour. The procedure is based on the assumption that bound water does not alter the freezing point of a given solution of sucrose, and therefore if a weighed quantity of bacterial cells with a known water content, as determined by desiccation of a portion of a uniform sample, is added to a sugar solution, changes in the freezing point of the solution will be due to the unbound water; the difference between the amount of water which affects the freezing point of the solution and the total water content as determined by desiccation should represent water in a bound state. While the method employed is probably not sufficiently accurate to make the percentages of bound and free water found in a given cell suspension entirely reliable, it is possible to show that these errors will be similar in two determinations when conditions are controlled and that comparative data of significance may be obtained when suspensions of two types of cells are run under identical conditions.

METHODS

Suspensions of vegetative cells of *B. subtilis*, *B. mycoides* and *B. megatherium* were procured by growing the organisms on a medium of the following composition:

	grams
Glucose	2.5
Peptone	5.0
Sodium chloride	0.5
Dipotassium phosphate	5.0
Ammonium sulphate	2.5
Water	1000 ml.

Spores were obtained on a medium identical with that given above, except for the addition of 1.75 per cent agar. After harvesting, the cells were washed four times in distilled water and examined microscopically to determine the ratio of spore to vegetative cells. In all suspensions used the ratio of the desired type of cell to the other form was 200 to 1 or higher.

After thorough washing, the bacteria were blotted between silk and filter paper and prepared for sampling by being well mixed on a silk cloth. Representative samples were transferred to dry, weighed containers and one of these used to determine the water content while the others were used to measure the effect upon the freezing point of water or a standard sucrose solution. The total water content of the cells was determined by heating at 100–105°C. until a constant weight was obtained. This latter procedure was checked several times and shown to give consistent results.

EFFECT OF BACTERIA UPON THE FREEZING POINT OF DISTILLED WATER

By the addition of various quantities of a single mass of wet, vegetative cells of *B. mycoides*, the water content of which was determined, to known quantities of distilled water the effect of several concentrations of the cells themselves upon the lowering of the freezing point was obtained. After correcting for undercooling by using the tables of Harris (1925) it was possible to calculate the true freezing points of these suspensions on the basis of grams of solids per 100 grams of water. The results obtained are as follows:

BACTERIA	WATER	FREEZING POINT
grams	*grams*	°*C.*
0.6627	100	−0.018
1.1803	100	−0.022
3.1705	100	−0.030

If these points are plotted, using lowering of the freezing point against grams of organisms per 100 grams of water, a straight line relationship can be demonstrated. Because of this linear relationship the effect of any quantity of bacterial material upon the freezing point of water can be obtained.

DETERMINATION OF BOUND WATER IN BACTERIAL CELLS

Accurately weighed quantities of moist bacteria with a known water content were placed in distilled water and in sucrose solution. This sugar solution was prepared by adding 16.000 grams of sucrose, which had been dried three days over fresh calcium chloride, to sufficient water to make 100 ml. of solution. Density measurements by the pycnometer method were made and the concentration of sucrose was found to be 0.1507(5) gram for each gram of solution used. The weights of total water, bacterial solids and sucrose were determined and the freezing point for each mixture was established. Masses of spores and vegetative cells of a given species were run in the same manner and all solutions and procedures were as nearly identical as possible for the two types of cells.

As an example of the results thus obtained the figures for *B. subtilis* are given in table 1.

TABLE 1

The effects of B. subtilis spores and vegetative cells upon the freezing points of distilled water and sucrose solution

	VEGETATIVE CELLS 68.86 PER CENT WATER, 31.14 PER CENT SOLIDS		SPORES 72.38 PER CENT WATER, 27.62 PER CENT SOLIDS	
	Distilled water	Sucrose solution	Distilled water	Sucrose solution
Moist weight of bacteria	0.3693	0.4609	0.5072	0.5581
Weight water added	13.0532		14.0895	
Weight sugar solution added		14.6864		15.1076
Freezing point	−0.019	−1.034	−0.020	−1.066
Undercooling, degrees C	1.0	0.8	0.6	1.1

From these data, again using *B. subtilis* as an example, it is possible to obtain or calculate:

1. The dry weight of the bacteria in sugar solution:	
Vegetative cells	0.1435 gram
Spores	0.1541 gram
2. The weight of sucrose in the sugar solution:	
Vegetative cells	2.2139 grams
Spores	2.2775 grams
3. The total weight of water in the sugar solution:	
Vegetative cells	12.7898 grams
Spores	13.2340 grams
4. The weight of organisms per 100 grams of water:	
Vegetative cells	1.122 grams
Spores	1.165 grams
5. The corrected freezing points in sugar solution:	
Vegetative cells	−1.028°C.
Spores	−1.051°C.
6. The corrected freezing points in distilled water for quantities of bacteria equal to those used in sugar solutions:	
Vegetative cells	−0.024°C.
Spores	−0.022°C.

7. The weight of sucrose per 100 grams water:

Vegetative cells	17.3098 grams
Spores	17.2094 grams

By applying the formula developed by Gortner and his co-workers (Newton and Gortner, 1922) to these figures, it is possible to calculate the bound water present in the system. This formula is:

$$\frac{T-(t+K)C}{T-t} = \text{per cent bound water.}$$

where T = observed lowering of freezing point in sugar solution containing bacteria.
t = lowering of freezing point in distilled water plus bacteria (corrected).
K = constant calculated for lowering of freezing point of sucrose solution.
C = constant dependent upon concentration, related to amount of available water.

For first calculations the value of K was that of Sayre's (1932). The value of C may be calculated:

$$100 - \frac{(\text{grams sucrose per 100 grams water})\ (18)\ (6)}{342.2}$$

The percentage of bound water multiplied by the weight of the total water in the system gives the weight of bound water. In the following calculation the change in C due to water added with the mass of organisms is disregarded as insignificant.

Results obtained by this treatment of the data gave, in some cases, a negative value and for this reason a constant K^1 was used. K^1 was obtained by assuming 0.014° less lowering, for all concentrations of sugar, than the values observed by Sayre. This procedure is justified in that our interest lies in comparative rather than absolute values for the amount of bound water present in the various cell suspensions. Also as Sayre says: "This difference may be due to some systematic error in all freezing point measurements, such as purity of sucrose used, the degree of undercooling, or the calibration of the thermometer."

By similar treatment of data obtained when the vegetative and spore forms of *B. megatherium* and *B. mycoides* were used the water binding capacity of bacterial cell materials of the three species was determined. Table 2 gives results expressed as grams of water bound per gram of solids and also the per cent of bound water in the moist bacterial masses. These latter figures were obtained in the following manner:

(Grams solids per 100 grams moist mass)
(grams water bound per gram solid) = per cent bound water.

If the amount of water which may be bound by the solids in a given cell mass is subtracted from the total water present, the percentage of free water in *B. subtilis* vegetative cells would be 68.9, in spores 3.4; for *B. megatherium,* 60.2 and 4.5, and for *B. mycoides* 50.0 and 11.9.

TABLE 2

The bound water content of vegetative cells and spores of three bacterial species

	B. SUBTILIS		B. MEGATHERIUM		B. MYCOIDES	
	Vegetative	Spores	Vegetative	Spores	Vegetative	Spores
Grams bound water per gram solids	0	2.5	0.8	1.9	1.3	2.0
Per cent bound water in bacterial mass . . .	0	69.0	17.7	62.6	28.2	58.7

The thermal death time at 100°C. was determined for suspensions of spores of these three species which had been grown on the medium described above.

That a rather close correlation was found between the calculated free water and the relative heat resistance of the spores is shown below:

	FREE WATER	THERMAL DEATH TIME AT 100°C.
	per cent	*minutes*
B. subtilis spores . . .	3.4	6
B. megatherium spores . . .	4.6	4
B. mycoides spores	11.6	2

The free water, as determined by us, approximates the total water content which in the past has been assumed for spores in explaining their heat resistance on this basis.

Thermal death point determinations showed that the vegetative cells of all three species are destroyed at approximately the same temperature (50°C.). This would be expected if the work of Lewith (1890) on the effect of various concentrations of water on the coagulation temperature of albumin is accepted. Lewith showed that variations in high water concentrations did not materially affect the coagulation temperature of egg albumin, whereas low concentration differences markedly influenced the coagulation temperature.

SUMMARY

1. Bound water determinations, by the cryoscopic method, have been made on the vegetative and spore forms of *Bacillus subtilis*, *Bacillus megatherium* and *Bacillus mycoides*.
2. In all cases the spores were shown to have a far greater water binding capacity than did the vegetative cells.
3. The theory is advanced that the heat resistance of bacterial spores is due in part at least to the relatively high percentage of water in the bound state.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN MISSOURI BRANCH

SPRING MEETING, ST. LOUIS, APRIL 12, 1938

ATTEMPT TO SECURE FERMENTING VARIANTS BY SERIAL TRANSFERS IN SUCROSE. *MacDonald Fulton*, Saint Louis University School of Medicine, St. Louis.

Sucrose-fermenting variants failed to appear when a group of 30 strains representing 8 species of sucrose-negative *Escherichia* were transferred serially in sucrose broth according to the method of Tregoning and Poe (Jour. Bact. 1937, *34*, 465). Storage for 6 weeks at 37° and subsequent plating also failed to reveal fermenting variants. The statistical significance of these opposing results was examined and their bearing on the theory of dissociation discussed.

SOME EFFECTS OF CARCINOGENIC SUBSTANCES ON YEASTS. *C. W. Dodge*, Missouri Botanical Garden, St. Louis.

NOTES ON THE DIAGNOSIS AND TREATMENT OF TULAREMIA. *George A. Hunt and Wm. F. Friedewald*, City Hospital No. 1, St. Louis.

The intradermal injection of a formaldehyde-killed, saline suspension of *Pasteurella tularense*, together with the agglutination reaction and the opsonocytophagic test proved most useful for an early diagnosis of tularemia. The use of an intradermal injection of a specific antiserum, described by Foshay, as a diagnostic aid, deserves further study since a positive reaction with goat antitularense serum and a negative reaction with normal goat serum was always confirmed by other diagnostic tests. It is difficult, however, to obtain animals for immunization whose sera do not react with many normal individuals.

A modification of the opsonocytophagic test, which may be of some value to State diagnostic laboratories finding it difficult to obtain fresh blood specimens, consists of the use of equal volumes of fresh citrated normal blood, the serum to be examined, and a suspension of *Pasteurella tularense* or *Brucella* organisms.

In a study of fifty cases of tularemia over a period of two years (thirteen cases having been given serum within the first three weeks of the disease), the observations on treatment agree essentially with those made by Foshay.

A MANOMETRIC METHOD FOR THE EVALUATION OF ANTISEPTICS. *J. Bronfenbrenner, A. D. Hershey and J. A. Doubly*, Washington University School of Medicine, St. Louis.

The parasitotropic and organotropic properties of antiseptics were determined in the Warburg-Barcroft apparatus. Measurements were made of the effect of antiseptics upon the rate of oxygen uptake by *Escherichia coli* and adult mouse liver tissue respectively, as compared to the rate of oxygen uptake in absence of antiseptics as control.

The concentration of antiseptics which causes a 50 per cent reduction in the oxygen uptake in a given time interval is taken as an arbitrary end point. The results obtained with *E. coli* show that for the antiseptic investigated, the effective concentrations fall approximately half way between the final bacteriostatic and germicidal concentrations as determined by the test tube culture method.

For purposes of comparison it is possible to calculate a respirometer coefficient in terms of phenol or bichloride of mercury.

A toxicity index for each antiseptic can be calculated from the results obtained on liver tissue and *E. coli*. The relation between the two values gives an indication as to the therapeutic possibilities of the compound. The values so determined are being compared at present with the effectiveness of the corresponding compounds in *in vivo* tests. To date our experience has been limited to two test materials (mouse liver cells and *E. coli*). We hope, however, to extend this study to other tissues and organisms.

The method has an advantage in that the results are immediately available.

A SUGGESTED LABORATORY METHOD FOR TESTING FUNGICIDES RECOMMENDED FOR THE TREATMENT OF EPIDERMOPHYTOSIS. *E. Burlingame, and G. F. Reddish*, Lambert Pharmacal Co., St. Louis.

The following organisms are used: *Epidermophyton inguinale, Epidermophyton intergitale, Epidermophyton rubeum, Epidermophyton gypseum, Trichophyton rosaceum.* Each organism is streaked over the surface of Sabouraud's agar in a 9 cm. Petri dish, using a dry sterile cotton swab inoculated with a five-day culture of these organisms. These plates are incubated at room temperature for five days. The agar cultures are then cut into 1 cm. squares. The fungicide to be tested is then poured over the surface of the culture so as to entirely flood the plate. After 5, 15 and 30 minutes one of the squares of culture and agar is removed and placed into 10 cc. of sterile broth. The excess fungicide is washed out of the matted culture by shaking the tube lightly for two minutes. At the end of this time the block of culture is removed from the broth and streaked with the culture side down over the surface of a sterile plate of Sabouraud's agar. These plates are then incubated at room temperature for three weeks and observed for growth.

An effective fungicide should kill these test organisms within 5 minutes. Experience has shown that fungicides which kill the test organisms in 5 minutes by this test are effective in the treatment of epidermophytosis as determined by clinical test. It has also been proved that preparations which do not kill these organisms by this test within 30 minutes are not effective under clinical conditions. The time period selected should be between these two points and 5 minutes is suggested.

This test is simple, requires very little material, makes use of the organisms which are the most common cause of "Athlete's Foot", makes use of solid medium in which these organisms grow most luxuriantly, uses a large inoculum, and avoids satisfactorily any bacteriostatic activity of the fungicides tested. This method has been carefully checked by extensive clinical tests and found to be satisfactory for the purpose of indicating which fungicides will be effective under practical conditions of use.

THE QUEST OF THE ALASKA SEALSKIN. *Helen Norris Moore,* Fouke Fur Co., St. Louis.

A moving picture in color was shown depicting the annual expedition to the Pribilof Islands to take the U. S. Government sealskins. Scenes were shown of the flora of the islands, the life and habits of the fur seals, and the methods used in preparing the pelts for shipment to St. Louis for dressing and dyeing.

The bacteriological problems which were discussed included studies of salt tolerant organisms, effects of germicides, and routine counts of various liquors used during the process of preparing these furs.

DEGENERATION AND VARIATION OF GONOCOCCI

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Received for publication September 21, 1937

I. THE CULTURAL BEHAVIOUR OF DEGENERATING GONOCOCCI

In a previous paper (Casper, 1937a) we reported our investigations concerning the serological typing of 109 strains from acute cases, and some strains from chronic cases, of gonorrhea. Using two completely type-specific sera, we were able to classify a large number of the strains by means of comparative agglutination.

Several difficulties arose in the classification of the remaining strains. Some strains which we could not classify by comparative agglutination were also unclassifiable by the agglutinin-absorption test. At times, the agglutinin-absorption test gave inconsistent results even with our test strains used for immunization.

We also called attention to the fact that the cultivation of suitable test strains of gonococci is exceedingly difficult. In view of the lack of suitable culture media and of our incomplete knowledge of the optimum conditions for the cultivation of this organism, the danger of degeneration must always be considered. Experience has shown that degenerative changes in the cocci, which are not necessarily accompanied by a change in morphology, often lead to changes in their antigenic properties. Thus, the test strains with which diagnostic sera are prepared and which serve as controls in the specific absorption of strains under examination, offer numerous sources of error.

It is well known that the type-specific carbohydrate of the pneumococcus, which is always present in organisms freshly isolated from human cases and virulent for mice, may be lost rapidly upon cultivation on artificial media. It is also known that the loss of type-specific carbohydrate is accompanied not only by a loss of virulence for mice, but also by a loss of specificity in all immune reactions. Conversely, pneumococci which by mouse-passage have become very virulent for these animals, give strongly type-specific immune reactions. Thus, we have at our disposal a means of maintaining the type-specificity of these organisms. Griffith (1928) points out that virulent pneumococci are entirely insensitive to the antibodies of the avirulent cells (R-pneumococci) and, consequently, are unable to exhaust the agglutinins from an R-serum. He also showed that the R-variant was unable to remove the type-specific agglutinins of the mother-strain. The same author (1920) was able to split off a serological variant of a type-specific meningococcus. This variant, without showing any cultural signs of degeneration, could not remove the agglutinins from a type-specific serum. He directed attention to the fact that such cultures could give rise to diagnostic errors.

Schiemann (1929) reported on the occurrence of "pseudotypes" in pneumococci and meningococci. In agar cultures of old laboratory strains of meningococci, he found a characteristic variation in colony morphology. The colonies were very small and represented a new-serological type. These strains were not agglutinated by type I or type II serum, but only by the serum of a degenerated type I strain. This "pseudotype" he never found in strains freshly isolated by lumbar puncture. Sometimes, however, it was found in strains obtained from pharyngeal cultures of meningococcus carriers. The sera of such "pseudotypes" were, naturally, unfit for the diagnosis of cases which actually occurred in practice.

Atkin (1925) attempted a classification of the gonococcus on the basis of colony morphology. He found, in fresh cultures of acute cases of gonorrhea, large papilla-bearing colonies which he designated type I. After prolonged incubation of these, a small papilla-free variant was split off. This variant he called type II.

He considered it especially noteworthy that he was able to isolate strains identical with his type II variant from cases of chronic gonorrhea. These strains were found most frequently in the cervix. According to Atkin's observations, the small papilla-free colonies are overgrown soon after isolation by the large papilla-bearing colonies but again gain predominance in aging cultures. He claimed that the papilla-free organisms were an individual pathogenic type, and assumed that classification based on colony morphology was at least as valuable as classification by serological methods. He prepared a "type I" serum with a strain freshly isolated from a male patient and his "type II" serum with an old laboratory strain showing only papilla-free colonies and originally isolated from a cervix. With these sera he was able to distinguish: (1) early forms which gave no serological reactions, but which, on further development, reacted with his "type I" serum and after the splitting off of "type II" reacted with his "type II" serum, (2) "type I" strains, (3) "type II" strains, (4) strains reacting with both "type I" and "type II" sera. A few strains were not classifiable according to his serological methods.

These investigations appear particularly interesting if one considers our observations that, in chronic gonorrhea, we found numerous strains which reacted as strongly with our type I serum as with our type II serum.

Experimental

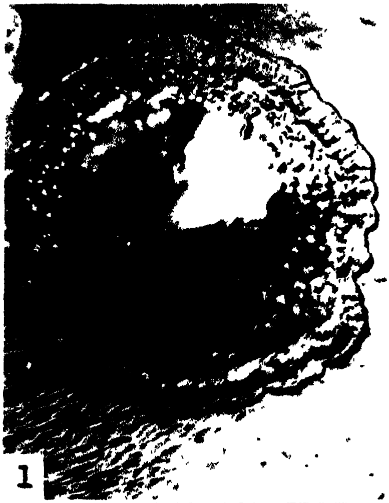
Atkin has stated that the degenerated forms can be observed only on a semi-solid pea-broth or horse-serum agar at a pH of 7.8. While optimum conditions for growth are provided on horse serum or ascitic agar at a pH of 7.5, the gonococci tend to autolyze because of rapid growth and, as a consequence, die before the formation of papillae has begun. A pH of 7.8, on the other hand, delays growth, impedes autolysis and thus creates the conditions conducive to degeneration.

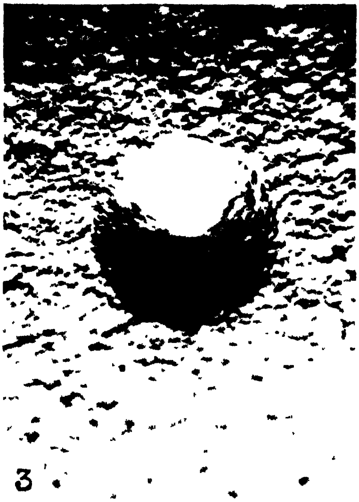
In our experiments we closely observed several strains of gonococci which had been cultivated for a long time. After testing a large group of media, we found that the best results were obtained with our blood-water agar (Casper, 1929). On this medium, which has a pH of 7.5 to 7.6, large single papilla-bearing colonies were formed, so that it appears as if the semi-solid state plays a more important rôle than the pH. After three to five days on this medium, transplants could still be made from every portion of the colony. According to Atkin, degeneration proceeds in the following manner: At the time of cultivation on alkaline medium, a group of papillae are formed on the colony. Transplantation after five days reveals that only the papillae-bearing portions of the colony are living. Lenz and Schaefer (1936) assume that the papillae are centers of regeneration of the colony while all the other parts of the colony are dead.

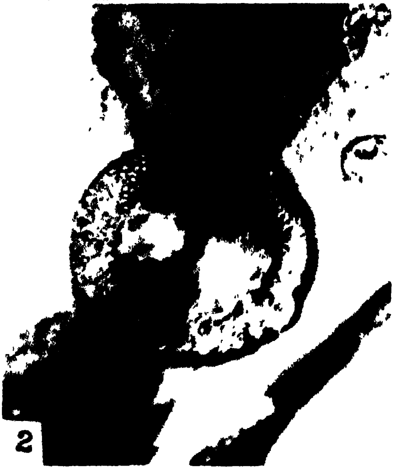
We did not find dissociation of papilla-free from papilla-bearing colonies so simple. In our transplants, firstly, the papilla-free portion was not dead and, secondly, papilla-bearing colonies like those with which we started were obtained from both the papilla-free portions and from the individual papillae. In the first cultures, some completely smooth colonies were found after transplantation. These, however, proved to be non-viable, and we were not able to obtain pure papilla-free cultures in this way.

In order to obtain papilla-free colonies, we turned to culturing the papilla-free central part of the colony which, according to Atkin, is supposed to consist of confluent papillae. By this means, we obtained some colonies which produced only a few papillae after numerous transplants. As a rule, transfers were made after 4 to 5 days growth in the incubator at 37°C. By transferring only the smooth part of the colony, we eventually obtained pure papilla-free cultures. The smooth part of the colony was triturated in saline and one drop of a 1:100,000 dilution placed on a blood-water agar plate and spread with a platinum spatula, so that as many and as large colonies as possible might be obtained.

The original plate of the test strain, transplanted simultaneously, exhibited the same characteristics as on the first day. In the case of our type I gonococcus, the first transplants showed some colonies with both mesial and marginal papillae (fig. 1).







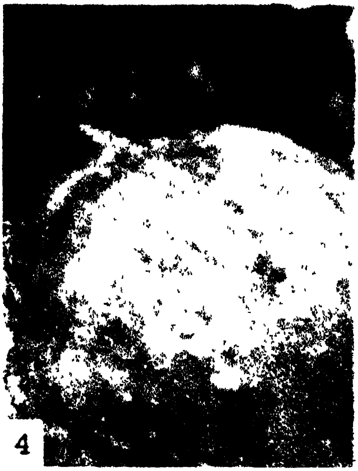


Fig. 1 Specific Colony with Papillae, and Flat, Convoluted Borders

Papillae both marginal and central

Fig. 2. Papilla-bearing Colony after Cultivation and in the Process of Transformation to the Smooth Variant

Papillae only marginal, center almost completely smooth, border no longer convoluted but almost round

Fig. 3 Typical Smooth Form

Colony has no border, conic, completely smooth, no papillae. The apparent irregularities are merely the reflexion of the rough surface of the agar.

Fig. 4 Variant of the Smooth Form

Colony concave (center has sunk) Border quite round. No papillae.

These papillae became very small and marginal only, then finally disappeared (fig. 2). In the subcultures, which showed only papilla-free colonies, there occurred a split into colonies of two different sizes. One, a small hemispherical (fig. 3) and the other, a more concave colony which was larger than the first (fig. 4).

Papilla-free colonies were picked out of mixed cultures for the initial injections in the production of antisera. Later the pure papilla-free cultures could be used. The procedure for preparation of the antisera has already been described (Casper, 1937a).

The transformation of our type II gonococcus culture followed a similar course. In this case, as in type I, most of the colonies also had numerous papillae and there were a few papilla-free colonies to begin with. These, however, proved to be non-viable and transplants were made from the papilla-free portion of a papilla-bearing colony. Type II also underwent several reversions to the papilla-bearing form. Strangely, there appeared large, papilla-free, staphylococcus-like whitish colonies, which, however, proved to consist of gram-negative bean-shaped diplococci. As in case of type I, we first had to use the papilla-free colonies of suitable cultures for the immunization of rabbits. Later, we obtained serviceable papilla-free cultures.

The papilla-free colonies appeared sooner than those of type I and two modifications arose, large and small smooth colonies. By subculturing, we obtained both of them in pure form.

We found no differences in the gram-staining properties of the gonococci in the original culture, in the concave papilla-free colonies, or in the large and small papilla-free colonies. We never observed those gram-positive gonococci reported by some authors (Raven 1934, etc.).

In similar fashion, a number of other gonococcus cultures which had been cultivated over a period of several months to over a year, produced both types of colonies, with and without papillae, on our blood-water agar. In every case we were able to obtain both forms in pure culture. This, however, required about three months after the original transplants had been made. We examined four each of our type I and type II and some of our overlapping strains. The papilla-free and papilla-bearing forms of the overlapping strains were not tested serologically.

Cross-serological reactions in reciprocal sera were now undertaken with the type I strain (98) and the type II strain (116). The results of the tests of these strains, with and without papillae, and the sera obtained from them are given in table 1.

From this table it is seen that we were unable to establish those sharp serological differences between the serological types and their variants which Atkin described. We must consider, however, that Atkin produced the "type II" serum used in his diagnostic experiments with laboratory strains which had been cultivated for two years, and that he was not completely successful in verifying, by serological examination, the derivation of his "type II" from a "type I" strain. Similar to our findings, he could only describe an increase in the related reactions, which in our experiments, were most pronounced with the smooth variant.

To us, however, it seems most important that this table clearly shows that type II, as far as regards its ability to form papillae as well as its serological specificity, is to be considered as an individual type and not a derivative of type I transformed by degeneration.

Since immunization of our rabbits was begun at a time when our strains had not yet completely lost their ability to form papillae, the experiment had to be enlarged so that more exact knowledge might be gained as to how great the serological differences due to degeneration may become. Nevertheless, our results show that degenerated strains are more sensitive to overlapping antibodies.

According to the prevailing conception, we must assume that, as in the case of pneumococci, the type-specific carbohydrate masks the remaining antigenic valencies of the gonococcus.

In this experiment it would have been possible to classify our papilla-bearing type I strain on the basis of its reactions with the antisera of the degenerated variants of both types. Classification of the type II strain, however, could not have been reached on this same basis since its reactions with the sera of the degen-

TABLE 1

Cross agglutination tests of the typical (papillae-bearing) strains of types I and II and their smooth variants with the corresponding antisera

STRAINS TESTED	NORMAL RABBIT SERUM			ANTISERUM OF THE PAPILLAE BEARING STRAIN 98						ANTISERUM OF THE SMOOTH VARIANT OF STRAIN 98						ANTISERUM OF THE PAPILLAE BEARING STRAIN 116						ANTISERUM OF THE SMOOTH VARIANT OF STRAIN 116					
	25	50	100	25	50	100	200	400	800	25	50	100	200	400	800	25	50	100	200	400	800	25	50	100	200	400	800
Strain 98 type I with papillae	0	0	0	++	++	++	++	++	+	++	++	+	±	±	−	±	−	−	−	−	−	±	−	−	−	−	−
Smooth variant of strain 98.	0	0	0	+	++	+	+	±	−	++	++	++	++	+	+	+	+	+	±	±	−	+	±	±	−	−	−
Strain 116 type II with papillae........	0	0	0	±	−	−	−	−	−	++	+	±	−	−	−	++	++	++	++	+	+	++	++	±	−	−	−
Smooth variant of strain 116	±	±	±	+	+	±	±	−	−	+	±	±	±	−	−	++	++	++	++	++	+	+	++	++	±	±	−

erated types were appreciably different. Apparently the smooth variants differed in the degree of their degeneration. This is seen from the fact that the smooth variant of type II gave a ± reaction in 1:100 normal serum while the smooth variant of type I gave a negative reaction.

We have not investigated the question as to whether there is a specific early form without papillae, which, as Atkin assumes, is responsible for the relatively frequent lack of reactivity between freshly isolated type I gonococcus strains and their homologous sera and which, after a short period of cultivation gives a type-specific reaction. On the basis of our serological experiments, however, there is no indication for the assumption of such a form.

The use of unclassified papilla-bearing strains for the preparation of antiserum seems to assure no absolute protection against the appearance of co-agglutinins. Despite this, it should be valuable to carry out a control of the cultural behaviour, on suitable media, of the strains which are to be used for immunization and for testing the serum. Of course, the demonstration of type-specific precipitating antibodies in diagnostic sera with the aid of the specific carbohydrates (Casper, 1937b) offers far greater certainty.

Summary

Strains from fresh cases of acute gonorrhea in the male and classified as two distinct serological types were transformable from the papilla-bearing to the papilla-free form.

The formation of papilla-free colonies is a sign of degeneration probably due to growth on artificial media. Diagnostic sera prepared with such degenerated cultures are very likely to give rise to errors in classification.

Classification based only on colony morphology does not serve to exclude the possibility that we may be dealing, not with different types, but with degenerated forms of the same type which have only lost their type-specific carbohydrate.

II. THE SEROLOGICAL BEHAVIOUR OF DEGENERATED GONOCOCCUS STRAINS

The difficulties arising in the classification of freshly-isolated gonococci (Casper, 1937a) caused by non-specific reactions with type-specific immune sera have been previously indicated. In order to clarify the question as to whether the test-strains with which our immune sera were prepared had degenerated, or whether the immune sera themselves had changed, we resorted to the agglutinin-absorption test.

By means of absorption we were able to classify some strains which had given overlapping reaction with our type-specific immune sera. No definite conclusions, however, could be reached by this method with strains from chronic gonorrhea since all these strains absorbed the agglutinins of both the type I and type II sera.

It is well known that pathogenic bacteria in artificial cultures degenerate easily, and, as has been shown in the case of pneumococci, lose their ability to produce the type-specific factor. In this way they become agglutinable by the antisera of a type which does not influence the undegenerated strain. For, every pneumococcus possesses, besides the type-specific carbohydrate, factors common to all types. These common factors cannot be demonstrated because, as the cell is intact, the type-specific factor masks the reaction. In fact, due to the precipitation of these overlapping parts of the antigen, autolyzed type-specific cultures react with the antisera of heterologous types. In the same way, a partially degenerated culture might show overlapping reactions. In such cases, therefore, the types could be differentiated from each other only by absorption test. But, in an absorption test, the question always arises as to whether the overlapping reaction was caused, providing that one works with type-specific test strains and test sera, by the common protein antigen or by the presence of an individual type-specific carbohydrate related to two different type-specific factors.

We observed the serological behaviour of gonococcus test strains, previously type-specific, but which, as a consequence of long cultivation, had undergone degeneration. A few strains had been cultivated over a period of several years. Thus, strain 1 (type I) and strain 25 (type II) were observed for six and five years respectively. From time to time, these strains were tested by agglutination in homologous and heterologous sera and also by agglutinin-absorption. Agglutination tests with one typical strain after degeneration are described in table 2.

These strains retained their specificity for a long time. Later, however, simultaneous with the drop in the titer of the homologous serum from 1:1600 to 1:400, there appeared strong agglutination in heterologous serum and also spontaneous agglutination.

TABLE 2

Changing serological behaviour of a type I gonococcus after long cultivation. Reversion to type specific agglutination by cultivation in citrated blood after apparent complete degeneration

STRAIN 1 (TYPE I)	TYPE I SERUM	TYPE II SERUM	NORMAL SERUM	SALINE
At the time of isolation, November, 1927	1:1600+	0	0	0
After cultivation, October 1930 . .	1:400+	1:400+	1:400+	±
After transplantation to citrated guinea pig blood	1:400+	0	0	0

We were able to eliminate the spontaneous agglutination in the following manner: The culture was transplanted on 14 successive days in bouillon, to which increasing quantities (10, 25, 50 per cent) of citrated guinea pig blood had been added. This strain now gave blood water agar cultures which did not agglutinate in either saline or normal serum. At this time we found practically no co-agglutination in heterologous serum.

In table 3 are seen the results of absorption tests with strains 1a and 25a, i.e., strains 1 and 25 after degeneration. These strains were tested with antisera prepared from them while they were in the degenerated state. At the same time, they were tested with the antisera of type-specific strains.

Table 3 reveals the following facts: A degenerated strain is capable of absorbing the agglutinins from a type-specific serum (tests 1, 2, 4, 5). Thus, it remains possible to classify type-specific strains even though degenerated strains be used for absorption.

The agglutinins of the serum prepared with a degenerated strain are not absorbed by a fresh heterologous strain (test 8). This shows that a serum prepared with a degenerated strain, which has lost most of its specific carbohydrates, may still retain some of its type-specific qualities. Although these degenerated strains can evoke a type-specific immunological response *in vivo*, they themselves are no longer capable of reacting type-specifically. This can be seen from the overlapping reactions between the degenerated strains and their reciprocal antisera (tests 10, 11, 13).

TABLE 3

Absorption tests with freshly isolated and old laboratory strains and their corresponding antisera

TEST NUMBER	TYPE SPECIFIC IMMUNE SERUM	STRAIN USED FOR ABSORPTION	EXAMINATION OF THE NON-ABSORBED SERUM WITH					EXAMINATION OF THE ABSORBED SERUM WITH				
			Type I strains		Type II strains			Type I strains		Type II strains		
			1a	76	25a	57	72	1a	76	25a	57	72
1	41 type I	1a	1,600	800	50		50	50	25	50		50
2	57 type II	1a	100			1,600		25			1,600	
3	57 type II	25a			1,600					800		
4	72 type II	25a			1,600		1,600			400		1,600
5	72 type II	25a					1,600					400
6	72 type II	72					1,600					100
7	72 type II	1a					1,600					1,600
8	1a type I	72	800					800				
9	1a type I	1a	800					200				
10	1a type I	25a	800					200				
11	1a type I	25a	400					200				
12	25a type II	25a			400					50		
13	25a type II	1a			400					50		
14	25a type II	72			400					50		

The degree of degeneration of any particular strain may vary from day to day. In a very degenerated state, it cannot absorb the agglutinins from a type-specific serum (test 3). At such time it would be unsuitable for use as a test strain. In a more specific state, however, the agglutinins are very strongly absorbed (test 1, 2, 4, 5). This demonstrates that a degenerated strain under certain unknown conditions may regain its type-specific factor.

Discussion

The gonococcus, like the streptococcus and pneumococcus, has been classified into several serological types. Upon cultivation on artificial media, these types undergo changes which induce the loss of their type-specific characteristics. Thus, it is seen that the behaviour of the gonococcus is quite analogous to that of the pneumococcus and streptococcus.

Investigations on the classification of streptococci (Griffith, 1934) have revealed the occurrence of certain types which are capable of absorbing the specific agglutinins from heterologous sera. Cultivation of a type-specific strain may so alter its agglutinability that it can be agglutinated, not only by its homologous antiserum, but also by a heterologous antiserum. This may occur even though there are no apparent changes in morphological or cultural characteristics. Similar observations have been made on the pneumococcus. Griffith (1928) and Schiemann (1929), have demonstrated the existence of cultural variants which have lost their type-specificity. Thus, S-organisms are not agglutinated by a serum prepared with an R-strain. It is well known that those changes which induce the loss of type-specificity of pneumococci are due to a loss of specific carbohydrate and are accompanied by a loss of virulence.

The peculiar behaviour of gonococci observed in agglutination and agglutinin-absorption tests might also be attributed to certain changes which affect their type-specificity, that is to say, the characteristic or function upon which their serological identity depends.

The loss of type-specificity which is responsible for such pronounced variations in serological behaviour is a phenomenon observed in the serology of many bacterial species which differ widely in morphology, antigenic composition and pathogenic action. In the light of these considerations it is easily understandable that difficulties must arise in the classification of long-cultured strains if one uses diagnostic immune sera which have not been tested on many freshly isolated strains. The fact that the so-called "pseudotype" described by Schiemann reverted into a known type after seeming to be a new one for some time, shows that freshly isolated strains must be tested in the establishment of the pathogenicity of any serologically "peculiar" type.

The loss of type-specific carbohydrate and the concurrent loss of type-specific immune reactivity is not a sudden one, but as we showed for the gonococcus (Casper 1937 b), proceeds slowly and gradually. It is, therefore, probable that at certain times a degenerating strain may possess sufficient carbohydrate for the recognition of its original type, while at other times it will give quite non-specific reactions. Along the same lines, we have seen that many reversions took place during our attempts to transform papilla-bearing to papilla-free strains, and that this transformation could only be accomplished after a very considerable number of transplants. These papilla-free forms are not capable of eliciting a type-specific immune response and the antisera prepared with them cannot be used in the classification of type-specific strains. Thus, one may say, that the loss of type-specific carbohydrate increases the ability to elicite type-specific immune response decreases. However, the 5-and 6-year old laboratory strains must have retained some of their original qualities so that at times they were able to give type-specific reactions. Whether all three changes; loss of type-specific carbohydrate, transformation to papilla-free forms and overlapping reactivity occur at the same time, we have not determined. Probability speaks for it.

If we recognize the fact that the type-specific antigenic complex is apt to be broken up before reaching the antibody-forming cells so that it becomes exceedingly difficult to prepare type-specific immune serum with them, the possibility must be considered of a change of the same degree of rapidity, but of reverse order. In other words, in the transference of gonococci, even from acute gonorrhea, to artificial media, such rapid degeneration may occur that they may not only give overlapping reactions but may also be agglutinated by normal serum or saline. That these are signs of degeneration can be seen from table 2. This may account for the relatively high frequency of non-type-specific, overlapping strains, isolated from acute cases of gonorrhea.

We must now consider still another possible source of degeneration. It is known that throat cultures of meningococcus-carriers (Griffith, 1920) have yielded strains serologically different from those obtained by lumbar puncture. These strains, for the most part, are not of any definite type, but correspond to an intermediate stage, i.e., their overlapping valencies are more pronounced than those of strains from acute infections. The strains which we isolated from chronic gonorrhea never were a definite type, but always showed overlapping valencies and agglutination in saline and normal serum. Thus, we may assume that the gonococcus, by adaptation to human tissue, undergoes the same degenerative process which we see after adaptation to artificial media. This degenerative process, including the decrease of type-specific carbohydrate, carries with it a decrease of virulence. We have seen that under certain conditions type-specificity may be regained and thus may explain the exacerbation of symptom-free infections.

Summary and conclusions

1. Associated with the loss of carbohydrate in old gonococcus cultures, there is a developing relationship between formerly heterologous strains.
2. Since, at the time of isolation of gonococci from acute cases of gonorrhea, one cannot estimate the degree of their degeneration, this fact may explain the relatively high frequency of overlapping strains.
3. A theory has been advanced that in chronic gonorrhea the gonococcus, by adaptation to the human tissue, may undergo the same degenerative processes that occur after prolonged cultivation on artificial media.

III. THE SLIDE AGGLUTINATION OF TEST STRAINS OF GONOCOCCI

In view of the relatively frequent occurrence of gonococcus strains which were equally strongly agglutinated by each of two type-specific sera (Casper, 1937 a) we decided to investigate the question as to whether it was also possible to split off colonies from type-specific strains which would react with the serum of the heterologous type.

It is well known that in the cultivation of the specific phases of paratyphoid bacilli, subcultures with mixed phases are found so that unless special precautions are taken (adequate serological controls and cultivation of pure phases) most of the sera are mixed phase (Andrewes, 1922). Despite the presence of the non-specific phase in freshly isolated strains these mixed-phase sera may be sufficient for differential diagnosis, since, in these sera, the non-specific phase is "masked" and, therefore, does not exert any influence on the agglutinins of the other phase.

Bearing in mind the behaviour of the paratyphoid bacilli, we examined the antisera of our type I and type II gonococci with the assumption that, in the cultivation of these organisms, a change of phase may eradicate the serological differences between the types.

Technique of Slide agglutination: The strains tested were definitely classified by the comparative agglutination method (with the exception of the degenerated strain 25a). After diluting them with saline to 1:100,000 so that large single colonies might be obtained, the cultures were streaked on blood-water agar plates. Following the procedure used with paratyphoid bacilli and Griffith's technique with scarlatinal streptococci (Griffith, 1926 and 1927) drops of each of the concentrated sera being examined were placed next to each other on a slide and a part of a single colony emulsified in each of them. The results were read immediately as to (1) rapidity and (2) intensity of floculation. Some were read with a magnifying glass, but the majority with the naked eye. The following grades were distinguished: ++, flaky (a few large flocculi); +, flaky (several, but smaller flocculi); (+), coarsely granular (numerous fine flocculi); ±, finely granular.

TABLE 4

STRAIN	TYPE	TOTAL NUMBER OF COLONIES TESTED	SPONTANEOUSLY AGGLUTINATED	IMMUNE SERA PREPARED WITH LIVING GONOCOCCI								IMMUNE SERA PREPARED WITH KILLED GONOCOCCI															
				Type I serum 83				Type II serum 84				Type I sera								Type II sera							
												75				76				57				25a			
				++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0
76	I	10	0	10	0	0	0	0	0	2	8	0	2	1	7	0	2	1	7	0	2	1	7				
76a Degenerated mixed culture		10	5	5	0	0	0					0	(5)	0	0	0	(5)	0	0	0	2	3	0				
76b Specific subculture		13	0	13	0	0	0									0	(5)	3	5	0	0	2	11				
57	II	12	0	0	8	0	4	0	8	0	4									12	0	0	0	0	6	0	6
Old strain 25a	II	5	0	5	0	0	0	0	5	0	0									0	5	0	0	0	5	0	0

The first two occurred rapidly, showing a specific phase reaction. The last two were not so striking. In a few cases the so-called specific colonies were transferred to new plates and examined again after 24 hours incubation at 37 degrees C. Table 4 is an excerpt of numerous experiments with many different strains and their corresponding antisera.

An examination of the table reveals that strain 76 reacts specifically with serum 83 (type I). Two colonies, moreover, react quite strongly with serum 57 (type II) while one colony gives a weaker reaction. Subcultures of the former colonies yielded strain 76a. Testing of this subculture demonstrated the continued presence of mixed-phasic properties, although it could still be identified as a type I. Five of the ten colonies agglutinated spontaneously. Strain 76b was derived from that colony which gave a ± reaction with serum 57. Agglutinations done with this strain proved to be type-specific.

The presence of mixed phases is easily seen with strains 57 and 25a. Despite this fact, strain 57 can be recognized as a type II. Strain 25a, on the other hand, due to its long cultivation, has reached such a state of degeneration that it is no longer possible to classify it.

Discussion

By means of the slide-agglutination test, we were able to reveal relationships between heterologous strains of gonococci which could not be seen by a titration of agglutinins. It must be concluded from this that there may be factors which are active in concentrated sera, but which cannot be detected in diluted sera. This phenomenon appears comprehensible in view of the important rôle which the colloidal state of the medium plays in the agglutination reaction.

Let us point once more to the fact that Griffith (1926, 1937) in his investigations on the classification of scarlatinal streptococci believed that he was justified in assuming the existence of different phases of the specific types. Furthermore, let us refer to earlier experiments of Griffith (1920) in which he examined a very large number of meningococcus-carriers and discovered the striking facts that (in contrast to their results with organisms from lumbar-puncture), in the organisms growing on the mucous membrane, type II was more frequent than type I; and that stages intermediate between both types, which seldom occur in cultures from lumbar puncture, were exceedingly frequent. At that time they assumed that they were dealing with a labile antigen which was able to develop in both directions. This theory approaches the conception of the change of phase.

Overlapping reactions in comparative agglutination have been seen, for the most part, in strains which have undergone degeneration. According to the relationships revealed by slide-agglutination, we must now consider the possibility of these relationships being due either to the loss of type-specific carbohydrate and consequent preëminence of a common protein antigen or to the existence of a carbohydrate related to each of two heterologous types (C-substance of Boor and Miller (1931)).

Atkin classified gonococci by cultural behaviour into two types. Type II, isolated from chronic gonorrhea, was designated as a papilla-free variant of his type I; the latter, isolated from acute gonorrhea, was characterized by papilla-bearing colonies. In view of the findings of Griffith and Scott with meningococcus-carriers and our experiments with gonococci, we think it advisable to consider Atkin's experience with chronic gonorrhea in a different light.

By means of comparative agglutination, we were able to classify a large number of gonococcus strains freshly isolated from acute gonorrhea into two heterologous types. We also demonstrated the existence of a completely type-specific carbohydrate for each of these (Casper, 1937b). Slide-agglutination tests performed with representative strains, on the other hand, detected the presence of mixed-phasic colonies. By picking individual colonies, we could obtain both mixed and specific phases of the same strain. Moreover, we showed that continued cultivation of a type-specific strain led to degeneration and overlapping serological reactivity due to the loss of its carbohydrate. In addition, each of the papilla-bearing type-specific strains could be transformed to papilla-free variants by prolonged cultivation. Similar to the condition in carriers of the meningococcus, it is probable that chronic gonorrhea is not caused by an individual pathogenic type of gonococcus, but rather that degenerative processes like those occurring after prolonged cultivation on artificial media, may take place due to the adaptation of the organism to its environment.

Inasmuch as Atkin used old laboratory strains for the preparation of his diagnostic type II antiserum, it must be emphasized that, as the above mentioned evidence for degeneration proves, errors in classification were bound to arise.

A number of the practically observed difficulties in classification by serological methods can be accounted for by 1) preparation of diagnostic sera with organisms which were not sufficiently specific and 2) rapid degeneration of the strains immediately after isolation or accidental transplantation of a degenerated colony in purification of the culture. Whether such errors in classification can be avoided by slide-agglutination or any other method cannot yet be answered from our experiments.

Further study of variability is necessary for the solution of the difficulties of serological analysis. Many phenomena are still unexplained.

SUMMARY

1. After cultivation on artificial media, strains of gonococci which were type-specific in the comparative agglutination test were shown by slide-agglutination to have mixed-phase colonies.

2. We have discussed the possible errors in serological classification which may be caused by the use of mixed-phasic cultures.

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STUDIES ON HEMOLYTIC STREPTOCOCCI

V. THE CHARACTERISTICS OF HUMAN AND ANIMAL STRAINS OF GROUPS A AND C

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Received for publication February 11, 1938

REVIEW OF THE LITERATURE

Within recent years, fermentation of trehalose and sorbitol and precipitin grouping according to Lancefield's technique have been found to indicate the human or animal source of hemolytic streptococci which formerly could not be distinguished.

Holth seems to have been the first to consider fermentation of trehalose and sorbitol for the differentiation of hemolytic streptococci. He included them in his studies together with 41 other test substances, and failed to note their significance. That discovery was made by Ogura whose data showed that lactose, trehalose and sorbitol are useful test substances for the differentiation of the streptococcus of strangles from streptococci of other animal and human sources.

Edwards extended the studies of Ogura. He studied 173 animal and 75 human strains of hemolytic streptococci. All of his human strains fermented trehalose, but not sorbitol; 159 (91.9 per cent) of his animal strains fermented sorbitol but not trehalose. Edwards concluded that sorbitol-fermenting strains of animal origin rarely, if ever, infect man.

A number of subsequent investigators have studied hemolytic streptococci in respect to fermentation of trehalose and sorbitol. Brown has recently summarized their results. He comments that in his survey of 1064 strains studied by various investigators, less than one per cent failed to be assigned to their proper serological group by fermentation of sorbitol and trehalose. Nevertheless there are in the literature reports of strains which ferment trehalose but not sorbitol and yet belong to group C. Ogura found trehalose-positive, sorbitol-negative strains among his animal strains; Edwards reported finding 7 such strains (4 per cent) among the 175 animal strains which he studied; Plummer found that among 418 strains of hemolytic streptococci which she studied, 9 (about 2.2 per cent) fermented trehalose but not sorbitol, and yet fell into group C. Hare reported that he had studied 47 strains which belonged serologically to group C although they fermented trehalose and not sorbitol. Minett reported finding 7 strains which fermented trehalose and not sorbitol among his 50 strains of hemolytic streptococci of animal origin.

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[1] The junior author was responsible for the serological identification of most of the strains of groups A and C.

Fermentation of lactose and sensitivity to phage B have also been used by previous investigators in classification studies on hemolytic streptococci. Holman used lactose fermentation in his grouping of streptococci by fermentation reactions. It was used in studies on hemolytic streptococci by Ogura; Edwards; Plummer; and Hare. Lancefield found that sensitivity to phage B filtrate was a character correlated with animal origin. In preceding papers of this series, sensitivity to nascent phage B was utilized as a character for the identification of species of groups A and C.

EXPERIMENTAL

In this paper data are presented which confirm the usefulness of trehalose and sorbitol for the identification of hemolytic streptococci of groups A and C; and data are presented to show the usefulness of sensitivity to phage B to give further information concerning strains of groups A and C. Lactose fermentation is also a useful character for the identification of certain species of those groups, although on the whole it is of less importance than the other mentioned tests.

From the results of our study of trehalose and sorbitol fermentations by hemolytic streptococci it became apparent that the usefulness of these two test substances is limited to the differentiation of strains of Lancefield's precipitin groups A and C. (and group E, which apparently is unimportant when pathogenic strains are concerned; it will be discussed further on). Other serological groups may be differentiated from groups A and C by various cultural and biochemical tests described by Lancefield; Lancefield and Hare; Hare; Hare and Maxted; Long and Bliss; Sherman.

Group B, which commonly causes mastitis in cattle, is distinguished by its appearance on blood agar plates, by a lower final pH in glucose broth (4.2–4.8) and by hydrolysis of sodium hippurate. It is also distinguishable from groups A and C by resistance to nascent phage B.[2] The strains of group B resemble human strains of group A in fermenting trehalose but not sorbitol.

Group D (the "enterococcus") is distinguished from groups A and C by a heavier, more opaque growth on blood agar; by more vigorous fermentation of test substances including trehalose and usually also sorbitol; by a lower final pH in glucose broth (4.2–4.8) and by growth on 40 per cent bile agar. The strains of group D are also distinguishable from those of groups A and C by their resistance to nascent phage B.

Group F (the minute hemolytic streptococci of Long and Bliss) is distinguished from groups A and C by slow and scanty growth on blood agar. It is also distinguished from groups A and C by lack of sensitivity to nascent phage B. Sorbitol is not fermented; trehalose may or may not be fermented.

According to Lancefield and Hare, and Hare, group G strains resemble group A strains closely in their biochemical reactions, including the ability to digest human fibrin and to produce a soluble hemolysin. The eight strains of group G in our collection, all of which were previously studied serologically by Dr. Lancefield or Dr. Hare, were found to be resistant to nascent phage B, a character which differentiates them from the strains of groups A and C.

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[2] It is unfortunate that the alphabetical designations of serological groups must be used in the same sentences with the alphabetical designations of the streptococcus bacteriophages. The phage designations bear no reference to sensitivity of serological groups.

According to Hare, who first described group H, the strains of this group may be distinguished from those of groups A and C by their distinctive appearance on blood agar, and by their inability to produce soluble hemolysin. They may be differentiated from strains of groups A and C also by their lack of sensitivity to nascent phage B, if the entire group conforms with two representative strains for which the writer is indebted to Dr. Hare. In fermentation reactions these two strains resemble the strains of groups A and C.

TABLE 1

Sensitivity to nascent phage B as a test for differentiating groups A, C and E from other groups of hemolytic streptococci

GROUP	NUMBER OF STRAINS IN THE COLLECTION*	NUMBER OF STRAINS STUDIED FOR PRECIPITIN GROUPING	SENSITIVITY TO NASCENT PHAGE B
A	362	108	+
C	191	114	+
E	3	3	+
B	44	6	−
D	28	6	−
F	10	10	−
G	8	8	−
H	2	2	−
K	4	4	−
Total	648	257	

* The grouping of some of the strains recorded in this column was by biochemical methods alone.

Group K was first described by Hare, who reported that he found only eight strains. Four of his strains were available for this study. They were found to be distinguishable from groups A and C by their resistance to nascent phage B. There is nothing distinctive in their fermentation reactions.

Table 1 summarizes the data which show that groups B, D, F, G, H and K may be distinguished from groups A, C and E by lack of sensitivity to nascent phage B. The three strains of group E, all of which were originally described by Lancefield, were isolated from certified milk. That group E is of little or no importance in studies of human streptococci is indicated by the fact that no strains of this group were found in studies of hemolytic streptococci from human sources made by Lancefield and Hare; Hare; Hare and Maxted. None other than Lancefield's three strains were found in our collection. Through the courtesy of Dr. Lancefield, her three strains of group E were available for the present study. According to sensitivity to nascent phage B they could not be differentiated from the strains of groups A and C. They were distinguishable from the strains of those groups, however, by their ability to ferment both trehalose and sorbitol, a combination of reactions not found in strains of groups A and C (see table 3).

From the above descriptions of the characters of groups B, D, F, G, H and K, it is obvious that fermentation reactions with respect to trehalose and sorbitol are useless for differentiation, and that if these groups are included with groups A and C in differential studies by means of fermentation reactions the data are confused.

Excluding the strains of the groups mentioned, there remained 556 strains of groups A and C in our collection which was made up group by group, largely of strains already studied by other investigators. The attempt was made to obtain a sufficient number of strains of every subgroup of A and C to make a comparative study. Hence there are higher percentages of strains of the more unusual types in our collection than would be likely to be found in routine isolations.

The strains of groups A and C cannot be differentiated by their manner of growth on blood agar, nor by their final pH in glucose broth. They are alike in lack of ability to hydrolize sodium hippurate, and in lack of ability to grow on bile agar.

The data on the fermentation of trehalose and sorbitol by the 556 strains of groups A and C are summarized in table 2. Of the 409 strains of groups A and C isolated from human sources, 405 (99.0 per cent) fermented trehalose but not sorbitol. Three failed to ferment either substance; one fermented sorbitol but not trehalose. Of the three human strains which failed to ferment either substance, one was from a throat following recent recovery from septic sore throat; one was from a blister which was a primary lesion in a case of surgical scarlet fever. The other was received with no history excepting that it was of human origin. The single human strain which fermented sorbitol but not trehalose was received from Hungary with a history of having been isolated from a scarlet fever throat. Since this strain is so unusual as a human strain, but agrees with animal strains, it seems probable that sometime there may have been some error in regard to it.

According to the data in table 2, animal strains are more diverse than human strains in their fermentation of trehalose and sorbitol. Among the 147 strains of animal origin with the characters of Groups A and C, 32 fermented neither substance, and 33 behaved like human strains in fermenting trehalose but not sorbitol. Eighty-two strains, or 55.8 per cent of the total number, fermented sorbitol but not trehalose.

TABLE 2

Fermentation of trehalose and sorbitol by hemolytic streptococci of groups A and C

FERMENTATION REACTIONS	HUMAN STRAINS		ANIMAL STRAINS	
	Number	Per cent	Number	Per cent
Trehalose – Sorbitol –	3	0.7	32	21.7
Trehalose + Sorbitol –	405	99.0	33	22.5
Trehalose – Sorbitol +	1	0.2	82	55.8
Trehalose + Sorbitol +	0	0	0	0
Total number.....	409	99.9	147	100.0

Among the thirty-two animal strains which fermented neither trehalose nor sorbitol, 26 belonged to the species *Streptococcus equi* described in the third paper of this series.

Among the thirty-three strains of animal origin which behaved like human strains in fermenting trehalose but not sorbitol, ten were Group A strains from the udders of cows associated with human epidemics of septic sore throat or scarlet fever. The remaining 23 were group C strains.

The most significant characteristics of the human and animal strains of groups A and C,—fermentation of trehalose, sorbitol and lactose, and sensitivity to phage B filtrate,[3]—are summarized in table 3, which includes less strains than table 2 because three odd strains were omitted. Table 3 includes all groups of two or more strains. The arabic numerals by which the groups are designated in table 3 are applied merely to facilitate the present discussion.

Table 3 shows that the majority of human strains (2) are group A strains with the characters trehalose +, sorbitol −, lactose +, phage B filtrate −. The large group with those characters includes *Streptococcus pyogenes* and *Streptococcus scarlatinae*, species which have been described in previous papers of this series, and also other subgroups.

Another smaller group of human strains (1) differs from the main group only in lack of ability to ferment lactose.

The main group of animal strains (7) is characterized thus: trehalose −, sorbitol +, lactose +, phage B filtrate +. Another important animal group (8), made up chiefly of *Streptococcus equi* strains, differs from the main group in lack of ability to ferment sorbitol and lactose.

Table 3 shows that there are in the collection a considerable number of human strains of group C which ferment trehalose but not sorbitol (4). Most of them were indistinguishable from the animal strains of the same characters not only according to the tests recorded in table 3, but also according to other tests applied, as described in the first paper of this series.

Our collection probably contains a higher percentage of the trehalose-fermenting strains of group C of human origin than would be ordinarily encountered in routine isolations in a hospital laboratory. There is evidence, however, noted further on, that they may sometimes become frequent agents of human disease within restricted localities, and for limited periods.

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[3] Note that nascent phage B was used to differentiate groups A and C from other serological groups and that it is phage B filtrate which was found useful to differentiate group A from group C.

The majority of trehalose-fermenting strains of group C fail to ferment lactose (4). Our interest was directed to non-lactose-fermenting strains because there are in the literature occasional reports of such strains being isolated from cases of human disease. According to Holman's classification they would be designated

TABLE 3

The characteristics of human and animal strains of hemolytic streptococci of groups A and C

GROUP NUMBER	HOST	NUMBER OF STRAINS IN COLLECTION	NUMBER OF STRAINS STUDIED FOR PRECIPITIN GROUPING	FERMENTATION OF			SENSITIVITY TO PHAGE B FILTRATE	NUMBER OF STRAINS IN THE GROUP	
				Trehalose	Sorbitol	Lactose		A	C
1	Human	28	28	+	−	−	−	28	0
	Animal	1	1	+	−	−	−	1	0
2	Human	320	68	+	−	+	−	68	0
	Animal	10	9	+	−	+	−	9	0
3	Human	8	7	+	−	+	+	2	5
	Animal	2	2	+	−	+	+		2
4	Human	46	35	+	−	−	+	1	34
	Animal	18	16	+	−	−	+	0	16
5	Human	0		−	−	+	+		
	Animal	3	2	−	−	+	+	0	2
6	Human	1	1	−	+	−	+	0	1
	Animal	1	1	−	+	−	+	0	1
7	Human	0		−	+	+	+		
	Animal	75	35	−	+	+	+	0	35
8	Human	0		−	−	−	+		
	Animal	29	8	−	−	−	+	0	8
9	Human	0		−	+	+	−		
	Animal	6	6	−	+	+	−	0	6
10	Human	3	3	+	−	−	−	0	3
	Animal	2	2	+	−	−	−	0	2
Total numbers...		553	224					109	115

Streptococcus equi. In the third paper of this series it was stated that the non-lactose-fermenting human strains examined did not agree with *Streptococcus equi.* Many of them, however, proved to be group C strains which fermented trehalose but not sorbitol. The human strains of group C were received from various sources, as follows:

Five strains were received through the courtesy of Dr. Griffith. They belong to three of his serological types, No. 7 (2 strains); No. 20 (1 strain); and No. 21 (2 strains).

Trehalose-fermenting strains of group C isolated from cases of erysipelas by Birkhaug were received from a number of laboratories. Birkhaug's experience with these strains is the most striking example of trehalose-fermenting strains of group C as an agent of human disease. He studied thirty-four strains of hemolytic streptococci isolated from typical cases of erysipelas. By means of the cross agglutination and agglutinin-absorption reactions he concluded that thirty-one of them belonged to one serological group. Believing that this group of streptococci was the chief agent of erysipelas, Birkhaug applied to it the name *Streptococcus erysipelatis.* Strains of this group were widely distributed in many laboratories in this country and Europe. Williams used one of them as the type strain of her erysipelas Type II, which included only six strains in her collection. In our collection of 45 strains from cases of erysipelas, the Birkhaug strains are the only group C strains. It appears, therefore, that he was misled in his conclusion concerning the importance of this group of strains as the agent of erysipelas, because he made his study at a time and place in which they happened to prevail.

Another lot of trehalose-fermenting strains of group C were received from Dr. Leonard Colebrook of Queen Charlotte's Maternity Hospital, London, England. Among the 36 strains of our collection isolated from cases of puerperal infection in the infectious ward of that hospital, 14 were trehalose-fermenting strains of group C.[4] They were all isolated during 1931 and 1932, when apparently a wave of group C puerperal infections occurred in London, similar to the wave of group C erysipelas infections which occurred in Baltimore in 1925. The group C strains seem to have disappeared from the London area before Dr. Dora Colebrook made her study of 121 puerperal strains from the same infectious ward, for she found no strains of Griffith's types 7, 20 or 21.

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[4] The names of the group C strains received from Dr. Colebrook are as follows: Jackson, Nash, Hewitt, Avery, East, Steele, Tavaris, Pickerin, Amelia Martin, Mary Martin, Keen, Cutbush, Sampson and Cuzwer.

A group of four strains of group C which fermented trehalose but not lactose nor sorbitol were received through the courtesy of Dr. Fisher, who had isolated them and five other similar lactose non-fermenting strains from cases of hemorrhagic smallpox during an epidemic in Detroit, Michigan.

Other trehalose-fermenting human strains of group C were received from scattered geographical sources and various human diseases. One was from a case of pneumonia; 1 from empyema; 1 from an abscess; 1 from a case of septicemia; 1 from a case of angina. Six were from normal throats. Two strains had histories of having been isolated from throats in cases of scarlet fever. The history of one of the scarlet fever strains (Griffith's strain "Angel," type 21) stated that it was associated with another streptococcus. It seems probable that the other group C scarlet fever strain may also have been associated with another streptococcus. At any rate there is no reason to conclude on the basis of one strain that the trehalose-fermenting strains of group C may produce an erythrogenic toxin.

The animal strains of Group C (4) which fermented trehalose but not sorbitol were from the following sources: Three were from cases of bronchitis in chicks; 5 were from cases of strangles in horses; 6 more were from other equine sources; 4 were from bovine sources, 1 of them coming from a case of acute mastitis; a few were from a variety of other sources.

Since the human and animal strains of group C which ferment trehalose but not sorbitol are indistinguishable, it appears probable that human infections with this group of streptococci may be contracted from lower animals, and *vice versa.* The occurrence of waves of human infections with trehalose-fermenting strains of Group C suggests a possible connection with bovine mastitis caused by strains of this group. The fact that certain group C strains are capable of attacking man complicates the identification of human pathogenic strains. In routine testing, strains belonging to group C should not be disregarded as nonpathogenic for man on the basis of serological grouping alone. Their fermentation reactions should also be studied.

The five groups described above include about 95 per cent of all human and animal strains of groups A and C. The remaining 5 per cent of strains are divided among 5 small variant groups.

A study of table 3 shows that fermentation of trehalose but not of sorbitol, and resistance to phage B filtrate is a character of Group A strains regardless of host origin. Strains characterized by fermentation of sorbitol but not trehalose, and sensitivity to phage B filtrate (7), and strains negative to all 3 fermentation tests and sensitive to phage B filtrate (8), are of animal origin. Apparently they are unable to attack man. The intermediary group C strains (4) with fermentation reactions characteristic of human strains and phage sensitivity characteristic of animal strains may attack either man or lower animals.

Table 3 shows at a glance that there is no correlation between lactose fermentation and serological grouping. The usefulness of lactose fermentation in the study of groups A and C is due to the supplementary evidence afforded by the lack of fermentation of lactose, a character useful particularly for the identification of strains of *Streptococcus equi* (8) and strains of the intermediary group (4) capable of attacking both man and lower animals.

On account of the method by which this collection was built up, it would be useless to attempt to express in percentages, the distribution of groups A and C in man and the lower animals on the basis of our data. Table 3 shows, however, that among hemolytic streptococci of groups A and C the great majority of human strains belong to Group A, and that the great majority of animal strains belong to group C.

In table 4, the data on 222 strains which were classified according to precipitin grouping are arranged to show the correlation of trehalose and sorbitol fermentation and of sensitivity to phage B filtrate with serological groupings.

It is important to emphasize that the group of 222 strains chosen for serological study included most of the strains which showed irregularities with respect to phagological or fermentation reactions on the one hand, and host source on the other hand, and that the group included only a small percentage of strains of the large groups in which all the determined characters agreed with those accepted as typical for the host species. This selection of strains was made because it was found that it is in the small irregular groups that discrepancies occur between phagological and serological reactions. Thus, only about 23 per cent of the main group (2) of human strains was examined serologically, because no irregularities were found in that group, whereas about 80 per cent of the strains of the intermediary group capable of attacking both man and lower animals (4) were studied serologically because trehalose-sorbitol fermentations indicated relationship with group A, whereas phage sensitivity indicated relationship with group C. Almost all strains of the small groups of 10 or less strains each were included in table 4 because it is in the strains of these groups that most of the irregularities occur.

TABLE 4

Correlation of characters with serological grouping

GROUP	PERCENTAGE OF STRAINS WITH REACTIONS AS FOLLOWS:					
	Trehalose		Sorbitol		Sensitivity to phage B filtrate	
	+	−	+	−	+	−
A.	100	0	0	100	2.8	97.2
C*.	53.6*	46.4	35.7*	64.3	90.4	9.6

* See the text for the explanation of the high percentages of atypical reactions.

In spite of the fact that the strains included in table 4 were made up largely of those showing irregularities of one kind or another, nevertheless this selection failed to reveal significant percentages of discrepancies in trehalose-sorbitol fermentation, sensitivity to phage B filtrate and serological grouping among the group A strains. The characters of group A strains were definite, with 100 per cent trehalose +; 100 per cent sorbitol −; and over 97 per cent resistant to phage B filtrate.

On the other hand, table 4 shows irregularity in the correlation of characters in group C. It may be pointed out that the percentages of irregularities are so magnified in table 4, by the manner of selection of irregular strains for study, that the majority of C strains included in the table were trehalose-fermenting strains. Nevertheless, even in this group selected to include a high percentage of irregular strains, 90 per cent of the C strains were sensitive to phage B filtrate. The object of the summary in table 4 was to show that among the strains of group C, sensitivity to phage B filtrate is more accurate than trehalose-sorbitol fermentation as an index to serological grouping.

SUMMARY

The serological groups A and C may be differentiated by sensitivity to nascent phage B from other groups of hemolytic streptococci with the exception of group E, which is not encountered in investigations of pathogenic hemolytic streptococci.

The present differential study includes 556 strains of hemolytic streptococci of groups A and C, 409 from human, and 147 from animal sources.

Fermentation reactions in trehalose and sorbitol broths offer useful data for the identification of strains of groups A, C and E. Lactose fermentation is also a useful character for the identification of certain species of groups A and C.

The human strains of group C and those of group A are alike in their fermentation reactions in trehalose and sorbitol broths. Of the total of 409 human strains of groups A and C, 99 per cent fermented trehalose but not sorbitol.

Animal strains of groups A and C are more diverse than human strains in their reactions in trehalose and sorbitol broths. Among the 147 strains of animal origin 32, including the 26 strains of *Streptococcus equi*, fermented neither substance; 33, including 10 strains of group A, behaved like human strains in fermenting trehalose but not sorbitol; and 82 strains, or 55.8 per cent of the total number, fermented sorbitol but not trehalose.

Group A strains are characterized by ability to ferment trehalose but not sorbitol, and by resistance to phage B filtrate. The majority of group A strains ferment lactose; a smaller subgroup differs in failing to ferment lactose.

The main subgroup of C strains is characterized by ability to ferment sorbitol and lactose but not trehalose, and by sensitivity to phage B filtrate.

Another important subgroup of C strains (*Streptococcus equi*) differs from the main group in failure to ferment all three test substances.

The strains of another sub-group of group C resemble human strains in their ability to ferment trehalose but not sorbitol and resemble animal strains in their sensitivity to phage B filtrate. They appear to be equally capable of attacking both man and lower animals.

The five mentioned groups include 95 per cent of all human and animal strains. The remaining 5 per cent of strains of our collection were divided among 5 small groups. It is in the strains of these small unimportant groups that most of the irregularities in the correlation of serological grouping with sensitivity to phage B filtrate occur.

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NUTRIENT REQUIREMENTS OF L. DELBRÜCKII IN THE LACTIC ACID FERMENTATION OF MOLASSES

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Received for publication February 11, 1938

Lactic acid has been made from such materials as corn, potatoes, molasses, sugar beets, glucose, and whey by fermentation with *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Streptococcus lactis*, *Lactobacillus delbrückii*, or other organisms (See references at end of paper). The manufacturer's choice of raw material depends upon his location, equipment, the price of the carbohydrate, and the quality of the product desired. Choice of culture depends upon the raw material and the conditions of fermentation selected.

Of the widely available, directly fermentable raw materials, molasses is cheap, and *L. delbrückii* is perhaps the most useful culture for the transformation of its sugar to lactic acid. We have chosen this combination and determined the conditions producing the largest yield in the shortest time.

EXPERIMENTAL PROCEDURE

The cultures, obtained from distiller's malt, were maintained on a 10 per cent malt mash, with transfers made every three or four days. Titratable acidity of this seed mash should be the equivalent of at least 15 cc. of 0.1 N acid per 10 cc. Crude cultures appeared to give as good results as pure cultures.

The inoculant for the final mashes was grown for 24 hours in molasses mash containing 5 per cent sugar, with half the usual $CaCO_3$ and a slight excess of nutrients over those of the final mashes. The latter were seeded with 3 per cent of the inoculant.

Eight liters of final mash was fermented in a 9.5 liter Pyrex bottle equipped with a mechanical agitator, and also with a tube for moderate aeration of the fermenting mash. The theoretical amount of calcium carbonate for the complete neutralization of a 95 per cent yield of lactic acid was added at the time the mash was made up. All mashes were steamed for 90 minutes.

It was found that an incubation temperature of 50–52°C. was as high as could be used for good yields. There was no apparent trouble with infection in this range.

ANALYTICAL METHODS

Acids were determined by acidifying 300 cc. of the fermented mash with 10 cc. of concentrated sulfuric acid. After the sample had cooled and the calcium sulfate had settled, a 5-cc. clear, top sample was taken and continuously extracted with ethyl ether for 5 to 7 hours. This extraction removed both the lactic and volatile acids, which were titrated with 0.1 N NaOH and phenolphthalein as indicator. A 100-cc. sample of the clear, top liquid was taken for the volatile acid determination. It was diluted to 200 cc. in a Kjeldahl distilling flask and distilled down to 40 cc. This dilution and distillation were repeated five times. The distillate was then made up to 1000 cc., and a 100 cc. aliquot was titrated with 0.1 N NaOH. One-half of this titration was subtracted from the ether extraction titration, and the lactic acid content of the 5 cc. sample was computed from the remainder.

The accuracy of this method was tested by neutralizing 27 grams of lactic acid with 15 grams of $CaCO_3$, diluting to 300 cc., adding 10 cc. of concentrated sulfuric acid and proceeding with the analysis as with the samples. The extracts from three 5-cc. clear, top samples gave an average titration of 48.4 cc. of 0.1 N NaOH, which indicates complete recovery.

Too great an excess of sulfuric acid and a longer extraction will result in some of the H_2SO_4 being carried over with the lactic acid. Sulfuric acid should be tested for in the ether extract with barium chloride.

Fermentations were analyzed for ethyl alcohol by distilling 150 cc. from 250 cc. of fermented mash and determining the specific gravity of this distillate. However, in most cases no ethyl alcohol was found.

Residual sugars were determined by inverting a 5-cc. sample with HCl, and analyzing for reducing sugar by the macro Shaffer-Hartmann method (1921).

TABLE 1

Fermentation results

NUMBER	MASH SUCROSE CONTENT	NITROGENOUS NUTRIENT†	TIME OF FERMENTATION	RESIDUAL SUGAR AS SUCROSE	VOLATILE ACID AS ACETIC	LACTIC ACID YIELD ON SUCROSE
	grams per 100 cc.		*days*	*grams per 100 cc.*	*grams per 100 cc.*	*per cent*
1	9.20	14 per cent malt sprouts	3½	0.59	0.09	95.6
2	9.16	8 per cent malt sprouts 4 per cent steep water‡	4½	0.40	0.07	96.0
3	9.58	8 per cent steep water 10 volumes per cent thin grain residue§	6	0.30	0.07	95.0
4	9.15	14 per cent steep water	3½	0.45	0.01	94.5
5	9.28	8 per cent malt sprouts 10 volumes per cent thin grain residue	4½	0.40	0.08	95.4
6	11.22	8 per cent malt sprouts 10 volumes per cent thin grain residue	5½	0.62	0.09	94.0
7*	9.30	8 per cent malt sprouts 10 volumes per cent thin grain residue	3½	0.47	0.10	96.2
8	9.30	20 volumes per cent thin grain residue	6½	0.59	0.12	88.1
9	9.55	Nil	10½	1.98	0.23	71.3

* Blackstrap molasses; all others cane syrup.

† Expressed as per cent on weight of sucrose or as volume per cent in mash.

‡ The steep water used contained 50 per cent solids, about half of which was protein matter.

§ Thin grain residues contained 2.3 grams per 100 cc. solids.

FERMENTATION DATA

Various substances were tested as accessory nutrients for the fermentation of cane syrup: malt sprouts, steep water, thin grain residue, corn gluten meal, ammonium phosphate, ammonium sulfate, superphosphate, and beet molasses. However, the first three of these materials were found to give the best results and were used for most of the work.

Table 1 shows the data obtained with these three accessory nutrients. The kind, amount, and combination of these accessories will influence the yield and time of fermentation. The soluble, organic nitrogen in steep water probably explains the short time of 3½ days obtained in No. 4. The blackstrap molasses used in No. 7, which also finished in 3½ days, has more soluble nitrogen than the cane syrup used in the other runs. The results of No. 8 and particularly No. 9 indicate that a lack of accessory nutrients greatly decreases the yield and materially increases the time of fermentation. These accessories may also contribute biologically useful stimulatory substances (Snell, Tatum and Peterson, 1937).

The analyses show that only traces of the fermented sugar went to other products than lactic acid. A low residual sugar and not more than a trace of volatile acid are aids in the recovery of the lactic acid.

SUMMARY

Good yields (94 to 96 per cent) and short periods of fermentation (3½ to 6 days) were obtained in the lactic acid fermentation of molasses using *Lactobacillus delbrückii* and accessory nutrients such as steep water, thin grain residue, or malt sprouts. The accessories are thought to supply soluble, organic nitrogen and biologically useful stimulatory substances.

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THE ABSORPTION OF STAPHYLOCOCCUS BACTERIOPHAGES BY ENTEROCOCCI

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Received for publication February 23, 1938

The absorption of whole bacteriophages as well as of "pure line" types by the use of heat-killed susceptible and partially resistant strains of bacteria and extracts derived from such cultures has for some time attracted our attention (Rakieten, 1936, 1937). In the main, the results, paralleling those of Levine and Frisch (1934), Burnet (1934), and Sertic and Boulgakov (1935), demonstrated a marked capacity for phage absorption by susceptible organisms and their extracts. Cultures of susceptible strains of staphylococci when they were heat-killed, or of watery extracts prepared from such cultures possessed phage-inactivating action. Secondary cultures of *Salmonella enteritidis* inactivated only those types of the whole phage possessing some activity toward them. A few exceptions have been noted. Levine and Frisch, as well as Burnet, have observed the occurrence of a phage-susceptible strain whose extract had no phage-inactivating action. We have encountered a strain of *Staphylococcus albus* that absorbs bacteriophage during its growth period, but whose extract is devoid of phage-absorbing quality. Absorption of staphylococcus phages by some strains of *Bacillus subtilis* also constitutes another exception (Rakieten, 1937). The present report deals with the enterococci and their ability to absorb staphylococcus bacteriophages in spite of the fact that such organisms are not lysed by these lytic agents.

The term "enterococcus," coined by Thiercelin (1899) to designate certain Gram-positive, aerobic, fecal streptococci, now includes a fairly large group of organisms which has been defined more clearly by the studies of Andrewes and Horder (1906), Weissenbach (1918), Dible (1921), Meyer and Schonfeld (1926), Bagger (1926), Weatherall and Dible (1929), and Sherman (1937). These streptococci are all relatively thermo-resistant, withstanding a temperature of 60°C. for thirty minutes; they grow well on media containing 10 per cent to 40 per cent bile; they ferment glucose in broth with the production of a low final pH; and they are able to split aesculin in nutrient broth as well as in media containing bile. Glycerol, maltose, mannitol, lactose, salicin, sorbitol, and trehalose are fermented; raffinose and inulin are not usually attacked. Arabinose may or may not be fermented. Methylene blue in milk is reduced. Sodium hippurate may or may not be hydrolyzed. Other criteria have been added to aid classification, but the characteristics listed above are generally deemed sufficiently inclusive to permit identification.

The 16 strains of enterococci used in this study possess in general the properties listed above.[1] Strains Z-1 and Z-11 were isolated from blood cultures of two fatal cases of subacute bacterial endocarditis. These two strains are similar in every respect to *Micrococcus zymogenes* as originally described by MacCallum and Hastings (1899), although they are not hemolytic. Strains Z-2 and Z-3 were obtained from Miss Ruth Thomas of the Department of Bacteriology, Cornell Medical School. Strains Z-4, 5, 6, 7, and 8 came from the American Type Culture Collection and correspond respectively to their *Streptococcus liquefaciens* #4532, 4533, 4534, 4535, and 4536. They were isolated from tonsils, feces, and from the normal cervix and vagina. Culture E-9 is a beta-hemolytic Gram-positive diplococcus isolated from a normal stool. E-16 was isolated from material removed at proctoscopy from a case of ulcerative colitis; E-40 from prostatic secretion; E-68 and E-69 from enteric contents obtained during proctoscopy; E-94 from a stool culture from a case of mucus colitis, while S-9 was recovered from loose milk. The biological properties of these organisms are listed in table 1.

According to the criteria listed by Sherman (1937) the distribution of the above-mentioned strains among the possible species of the enterococcus group would be as follows: *Streptococcus fecalis*, E-16, E-40, E-9 and E-94; *Streptococcus liquefaciens*, Z-1, Z-2, Z-3, Z-6, Z-7, Z-11, and E-68; *Streptococcus zymogenes*, Z-4, Z-5, Z-8, and E-9; *Streptococcus lactis*, S-9, and possibly also E-69

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[1] These strains are part of a collection of Dr. E. J. Tiffany and have been identified by him.

TABLE 1

Biological reactions of enterococcus group organisms

STRAINS	SURVIVE MOIST HEAT		GROWTH ON MEDIUM CONTAINING			GROWS AT pH		FINAL pH IN GLUCOSE BROTH	REDUCES METHYLENE BLUE IN MILK	LIQUEFACTION OF GELATIN	PROTEOLYSIS OF MILK	HYDROLYSIS OF Na HIPPURATE	HAEMOLYSIS ON BLOOD AGAR	SOLUBLE HAEMOLYSIN PRODUCED	SPLITS AESCULIN IN BILE MEDIUM	FERMENTS WITH PRODUCTION OF ACID												
	60°C. 15 min.	60°C. 30 min.	40 per cent bile	4 per cent NaCl	6.5 per cent NaCl	9.2	9.6									Aesculin	l-Arabinose	Glycerol	Inulin	Lactose	Maltose	Mannitol	Raffinose	Salicin	Sorbitol	Sucrose	Trehalose
Z-1 .	+	+	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-2 . .	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-3 . . .	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-4 . .	+	+	+	+	+	+	+	4.6	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-5. . .	+	+	+	+	+	+	+	4.8	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-6 . . .	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-7 . .	+	+	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-8 . .	+	+	+	+	+	+	+	4.6	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-11 . .	+	+	+	+	+	+	+	4.8	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	−	+
E-9 .	+	+	+	+	+	+	+	4.6	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-16. . .	+	+	+	+	+	+	+	4.6	+	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-40.	+	+	+	+	+	+	+	4.6	+	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-68 .	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-69. .	+	+	+	+	−	+	∔	4.6	+	−	−	−	−	−	+	+	+	+	−	+	+	+	−	+	−	+	+
E-94. .	+	+	+	+	∔	+	∔	4.4	+	−	−	−	−	−	+	+	+	+	−	+	+	+	−	+	−	−	+
S-9 . . .	±	−	+	+	∔	+	∔	4.5	+	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	−	+	+

± = many viable organisms present but greatly reduced in numbers as compared with the non-heated control.

∔ = growth occurs, but is delayed and scanty.

and E-94. For testing the ability of these organisms to produce an area of hemolysis around a colony on blood agar, both human and rabbit bloods were used. Four strains were found to be hemolytic, the reaction being the same on both blood agar plates. These hemolytic organisms were also tested for their ability to produce the so-called soluble hemolysin. The technique used was a modification of that described by Hare and Colebrook (1934). Our tests were carried out with a serum-free twelve-hour broth culture of the organisms and a suspension of washed human red cells. None of the four cultures produced a soluble hemolysin under these conditions.

The two races of staphylococcus bacteriophage used have been employed in this laboratory for other investigations dealing with absorption. Phage "V" is monovalent, lysing only culture *Staphylococcus albus* "v." Phage M-16 is polyvalent and lyses both susceptible cultures used as host strains to approximately the same titer and within the same period of time.

Susceptible culture Vnt is a hemolytic *Staphylococcus aureus* strain isolated from a case of osteomyelitis. It is susceptible to phage M-16. Culture "v" is a non-hemolytic *Staphylococcus albus* strain and is lysed by both phages. Unless stated otherwise Savita broth and agar (Rakieten, 1932) were used throughout all of these experiments, since they support the growth of enterococci as well as staphylococci.

EXPERIMENTAL

Absorption of staphylococcus phages by living cultures of enterococci

To tubes containing 10 cc. of broth, 0.1 cc. of an eighteen-hour broth culture of enterococcus and 0.05 cc. of phage M-16 was added. (The bacteriophage was so diluted that, providing no absorption took place, plaques could be counted when approximately 0.02 cc. of the filtrate was plated on susceptible cultures.) After an incubation period of from 18 to 22 hours at 35°C. the tubes containing mixtures of enterococci and staphylococcus bacteriophage, as well as the controls, were filtered through L_5 Chamberland candles. Of each filtrate 0.02 cc. was then plated for plaque counts. Plates were examined after incubation for 18 hours. Reduction in the number of plaques by an amount greater than 50 per cent was considered as positive evidence of phage absorption. The results obtained when several strains of enterococci were tested for their phage-absorbing capacity, following the procedure outlined, are listed in table 2 (A and B).

Experiments of a similar nature were carried out with strains S-9, E-69, E-94, E-68, Z-2, Z-3, Z-4, Z-5, Z-7 and Z-8 with results comparable to those listed above. From the results obtained (and many of these tests have been repeated several times using phage "V" as well as M-16) one may conclude that growing cultures of enterococci possess the ability to absorb staphylococcus bacteriophage. When cultures of enterococci that have been in contact with these phages are examined microscopically and then plated no difference can be detected between them and control cultures.

TABLE 2

CULTURE	BP. ADDED M-16	APPEARANCE OF TUBE	pH BEFORE FILTRATION	pH AFTER FILTRATION	PLAQUE COUNT CULTURE V
			A		
	cc.				
E-9	0.5	Turbid	5.2	5.2	1
E-9	None	Turbid	5.2	5.2	0
Z-11	0.5	Turbid	5.2	5.2	0
Z-11	None	Turbid	5.2	5.2	0
Z-1	0.5	Turbid	5.2	5.2	0
Z-1	None	Turbid	5.2	5.2	0
Broth	0.5	Clear	7.4	7.4	421

E-16	0.5	Turbid	5.2	5.2	0
Z-6	0.5	Turbid	5.2	5.2	3
E-40	0.5	Turbid	5.2	5.2	0
Broth	0.5	Clear	7.4	7.4	314

It may be observed from tables 2, A and B, that all strains of enterococci produce in Savita broth a marked acid reaction. In order to assure ourselves that the presence of such a reaction in itself is not responsible for the marked diminution in corpuscular count the following experiments were carried out.

A. Absorption of phage by living cells in buffered broth

Savita broth (10 cc.) whose final pH was 7.5 was buffered by adding 0.5 cc. of 10 per cent Na_2HPO_4. (This solution was sterilized by filtration through L_5 Chamberland candles.) To these buffered broth tubes 0.1 cc. of eighteen-hour broth cultures of enterococci along with 0.5 cc. of diluted phage M-16 or "V" were added. After an incubation period of 22 hours the reaction in all of the tubes was between pH 7.0 and 7.5. Filtrates of these culture-phage mixtures showed as marked a reduction in plaque count as those recorded with unbuffered media. This may be observed by the results indicated in table 3.

TABLE 3

Absorption of staphylocococcus phage M-16 by enterococci in buffered Savita broth

CULTURE	PHAGE M-16	REACTION pH	PLAQUE COUNT ST. V
	cc.		
E-40	0.5	7.1	1
Broth	0.5	Over 8	168
E-9	0.5	7.0	5
Broth	0.5	Over 8	217
Z-1	0.5	7.0	2
Broth	0.5	Over 8	187

B. Kinetics of absorption of phage by living cultures

In a previous report (Rakieten, 1937) it was demonstrated that a considerable amount of bacteriophage was absorbed by strains of *Bacillus subtilis* in a period of 5 hours at incubator temperature. In this connection it is interesting to note that cultures of *B. subtilis* which absorb staphylococcus phages scarcely lower the original pH of the Savita medium (pH 7.5) even after an incubation period of 58 hours. It was thought advisable to determine at what period during the growth of enterococci staphylococcus phages are absorbed. Strains Z-11 and E-40 were inoculated into broth along with the staphylococcus phage M-16. The tubes were then incubated and at the end of 5 hours and 9 hours, tubes were removed, shaken, and before filtration a pH determination was done, as well as a bacterial count. Following filtration, pH determinations and plaque counts were made. Table 4 represents the results obtained.

It is evident that culture Z-11 in a period of 5 hours at incubator temperature, and when the medium is still alkaline, is able to inactivate a considerable amount of staphylococcus phage M-16. Culture E-40, while it has approximately the same rate of growth as has strain Z-11, takes a little longer to bring about a significant reduction in plaque count. On the basis of the evidence obtained in the above experiments it is believed that the absorption of staphylococcus phage by enterococci is not related to a change in the reaction of the medium brought about by the growth of these cultures.

The results obtained thus far lead us to believe that enterococci have some protoplasmic component in common with staphylococci, in that it is able to combine with and absorb staphylococcus phages. The antigenic component of susceptible strains of staphylococci and cultures of *B. subtilis* which absorbs staphylococcus phages is remarkably heat stable, since autoclaved cultures possess this phage-inactivating quality. Hence, it was desirable to ascertain whether heat-killed cultures of enterococci still retained the absorbing power. Cultures of Z-11, E-40, Z-8, E-69, E-9, E-16, Z-4 and Z-7 were heated for one hour at 62–63.5°C., the organisms were resuspended in fresh broth and then staphylococcus phages were added. Following incubation the mixtures were tested for the presence of phage by plaque count. In no instance did we find that there was any significant reduction over that of controls. In this respect the component in these

TABLE 4

CULTURE	BP. ADDED M-16	INCUBATION PERIOD	REACTION pH	BACTERIAL COUNT	REACTION FOLLOWING FILTRATION	PLAQUE COUNT
	cc.	*hours*		0.1 cc. 10^5		
Z-11 ...	0.5	5	7.1	1018	7.1	41
E-40.	0.5	5	7.0	994	7.0	175
Broth	0.5	5	7.5		7.5	234
				0.1 cc. 10^6		
Z-11 ..	0.5	9	7.1	124	7.0	18
E-40	0.5	9	6.8	76	6.8	1
Broth	0.5	9	7.5		7.5	204

strains responsible for phage absorption is much more heat labile than it is in staphylococci. However, a bacteriophage ("Zy") which lyses these strains of enterococci is absorbed by these same heat-killed cultures. This latter finding is in keeping with the results obtained by other workers dealing with the ability of heat-killed susceptible cultures to absorb homologous phages.

Cultures were killed by other means in an attempt to learn whether such cultures would absorb homologous phages. Commercial formalin in a quantity sufficient to bring the final concentration to 0.5 per cent was added to fifteen-hour cultures of the organisms in plain infusion broth. Following a twenty-four-hour incubation period they were centrifuged, resuspended, and washed three times with physiological saline. After a third washing the organisms were resuspended in saline; sterility tests showed no viable organisms.

Cultures Z-1, Z-11, E-9, and E-40 were subjected to the action of formalin in the manner indicated above. To 5.0 cc. quantities of the washed sediments, resuspended in saline, as well as to the third and final saline washings of each culture, 0.5 cc. of staphylococcus bacteriophage "V" and enterococcus phage "Zy" were added. After incubation for 20 hours a portion of each mixture was overlaid on susceptible staphylococcus "v" and enterococcus E-40, in order to obtain plaque counts. The results appear in table 5.

That the bacteriophage inactivating effect of formalin-killed enterococci was not due to the presence of formalin is evident, since in no instance did the final wash-fluid reduce the plaque count. Toward the homologous active phage, formalinized cultures have a considerable degree of phage inactivation power. With a staphylococcus bacteriophage that is markedly inactivated by these cultures in their actively proliferating state, these same formalinized cultures cause a significant reduction in corpuscular count.

Enterococci killed by another method also retain their staphylococcus phage-inactivating power. This method entails grinding the bacteria in an apparatus similar to that employed by Mak (1933). Our apparatus was constructed so that two tubes of culture might be ground at the same time. Pyrex tubes 3.5 x 17.5 cm. containing approximately 150 quarter-inch stainless steel balls revolving at a speed of 300 revolutions per minute constituted the grinding chamber.

Cultures of staphylococcus Vnt and enterococcus E-40 were grown in Roux bottles. After an incubation period of 24 hours the growth in each bottle was washed off with 5 cc. of sterile distilled water. The mixtures were then placed in the sterile grinding chamber and after 48 hours of continual grinding (average temperature 20°C.), the material was removed and examined microscopically and culturally. In the case of the staphylococcus, only Gram-negative amorphous material was observed. The enterococcus material revealed on smear relatively few organisms retaining their Gram stain. However, on culture, viable cells were found. In order to free this ground suspension of most of the viable cells it was centrifuged and the supernatant removed. These supernatant fluids, in 0.5 cc. quantities were added to 0.5 cc. of bacteriophages "Zy" and "V." These mixtures were incubated at 20°C. for 24 hours, and without filtering were tested for their ability to produce plaques. A portion of each supernatant was also heated for one hour at 63°C., to these

TABLE 5

Absorption of staphylococcus and enterococcus phages by formalinized enterococcus cultures

CULTURE 5.0 CC.	THIRD WASHING FLUID 5.0 CC.	BP. ADDED "ZY"	BP. ADDED "V"	PLAQUE COUNT	
				E-40	Sta. V
		cc.	cc.		
Z-11		0.5	0.5	0	76
	Z-11	0.5	0.5	Cfl	200
Z-1		0.5	0.5	0	101
	Z-1	0.5	0.5	Cfl	200
E-9		0.5	0.5	109	81
	E-9	0.5	0.5	Cfl	200
E-40		0.5	0.5	0	91
	E-40	0.5	0.5	Cfl	200
Broth		0.5		Cfl	
Broth			0.5		217

fluids, equal quantities of phages were added, and following incubation at 20°C. they also were plated for plaques. The results are listed in table 6.

Similar results were obtained with ground material from enterococcus culture E-40. These ground preparations were kept in the ice-box for three weeks and again tested for their phage-inactivating power. No decrease in absorbing quality was observed. Toward homologous phages, material from heated as well as from unheated ground cultures has very strong absorbing power. Ground cultures of enterococci that have been heated lose this staphylococcus phage-inactivating ability. This result is consistent with that obtained when whole cells have been subjected to heat. Ground cultures of staphylococcus Vnt, unheated, also absorb enterococcus phage "Zy."

TABLE 6

SUPERNATANT GROUND CULT.	BP. M-16	PLAQUE COUNT	SUPERNATANT GROUND CULT.	BP. "ZY"	PLAQUE COUNT
	cc.			*cc.*	
Vnt. unheated	0.5	4	Vnt. unheated	0.5	150
Vnt. heated	0.5	0	Vnt. heated	0.5	Cfl
E-16 unheated	0.5	161	E-16 unheated	0.5	0
E-16 heated	0.5	400	E-16 heated	0.5	0
Broth	0.5	400	Broth	0.5	Cfl

During the course of this study we have also tested three other susceptible strains of staphylococcus for their enterococcus phage-absorbing ability. In all instances positive evidence was acquired. Also, cultures of *B. subtilis* which previously had been shown to absorb staphylococcus phage will also absorb enterococcus phage "Zy." Enterococcus cultures E-16, E-40, and Z-11 will also absorb to a high degree *B. subtilis* phage C-3. Pneumococcus types I, II, and III when they were tested for their phage absorbing activity had no demonstrable effect on our staphylococcus phages or enterococcus phage.

Extracts of strains E-16, E-40 and Z-11 were prepared according to the method originally described by Burnet (1934) in order to determine whether the component responsible for staphylococcus phage-absorption is present in such preparations. The clear watery extracts of the above mentioned cultures were added in 0.5 cc. amounts to staphylococcus phages. After an incubation period of 20 hours at 35°C., approximately 0.02 cc. of each unfiltered mixture was overlayed on agar-plate susceptible cultures, and the plates later observed for plaque counts. Extracts prepared and tested in this fashion do absorb homologous phages, but have no demonstrable effect on staphylococcus phages. Protocol 7 illustrates one of the results obtained.

No differences in results were noted when staphylococcus phage M-16 was used. It is obvious that extracts of enterococci prepared in this fashion lack phage-inactivating action toward staphylococcus phages. Since these extracts are prepared by allowing the cultures to autolyze at 57° to 58°C. for 48 hours it may be that this degree of heat is responsible for the loss in absorbing effect, since heating living as well as ground cultures at a temperature only slightly higher destroys the absorbing effect toward these same phages.

TABLE 7

Watery extracts from cultures E-40, E-16, Z-11 and their effect on homologous and staphylococcus phages

EXTRACT (0.5 CC.)	BP. 0 5 CC.	PLAQUE COUNT
		E-16
E-40	"Zy"	109
E-16	"Zy"	0
Z-11	"Zy"	11
Distilled water	"Zy"	Cfl
		Sta. v
E-40	"V"	Over 400
E-16	"V"	Over 400
Z-11	"V"	Over 400
Distilled water	"V"	Over 400

Attempts, using serological methods, have also been carried out in order to learn whether the component in enterococci that absorbs staphylococcus phages is similar to staphylococcus protein. These tests are still being followed, but thus far they have given no conclusive evidence that precipitin produced by injecting rabbits with formalinized enterococci reacts to any considerable degree with staphylococcus precipitinogen (protein fractions). These same fractions react with homologous precipitin to a high titer. This portion of the study will be reported at some later date in greater detail.

Finally, protein and carbohydrate fractions of enterococci and staphylococci have been prepared in order to see whether these fractions will absorb homologous and heterologous phages. The methods used to isolate these protein and carbohydrate preparations were similar to those reported by Lancefield (1925) for the streptococci.

The organisms grown in Roux bottles on the surface of extract agar, containing 0.1 per cent glucose, were suspended after 48 hours' incubation in a small volume of sterile distilled water. The organisms were washed twice with sterile distilled water, once with 95 per cent alcohol, and once with ether, and dried in a vacuum desiccator. The dried mass was ground in the ball-mill for 12 hours, at the end of which time there were no intact organisms to be found in a Gram-stained smear. N/100 NaOH did not suffice to bring the material into complete solution and we were forced to resort to N/50 NaOH. To this solution 10 per cent acetic acid was added until precipitation was complete. The precipitate was then centrifuged and the supernatant decanted and saved. The precipitate was redissolved in N/50 NaOH and reprecipitated with acid. This process was repeated three or four times and the acid supernatants saved and pooled. The precipitate was finally washed several times in water and dried in the vacuum desiccator. This product was labeled "P" A.

The combined acid supernatants were evaporated to about 1/10 volume in a stream of warm air, acidified with acetic acid in the cold, any precipitate discarded, and the acid solution kept in a boiling water bath for 10 minutes to precipitate coagulable protein, and then centrifuged. To the clear supernatant was added slowly ten volumes of 95 per cent alcohol. The resulting precipitate was dissolved in 5 to 10 cc. of distilled water. This solution was reprecipitated with alcohol and redissolved several times. The final precipitate was dried in the vacuum desiccator and labeled "C" A.

Other mass cultures of these organisms, grown in the same way, were treated according to the method of Avery and Goebel (1933) in the preparation of acetyl polysaccharide from the pneumococcus. For this fractionation the surface growths from twenty-seven Roux bottles were used, amounting to 270 cc. of bacterial suspension. This represented the accumulated harvests over a period of ten days, and was stored in the ice-box in the meantime, with the addition of 1/10,000 merthiolate to prevent contamination. To this suspension was added about 80 cc. of N/1 acetic acid and the mixture was heated in the Arnold sterilizer at 100°C. for thirty minutes. Upon cooling, the bacteria settled to the bottom of the flask in a granular precipitate. After standing in the ice-box overnight, the bacteria, plus any coagulated material, were removed by centrifugation and the supernatant, supposedly containing the polysaccharide, was stored in the ice-box. The sediment was washed twice and the washings added to the supernatant. The sediment was washed with alcohol, ether, then dried, ground in the ball-mill, dissolved in N/50 NaOH, and precipitated several times with acetic acid. The final dry precipitate was labeled "P" B.

The combined washings and supernatant were evaporated to 1/10 volume and to the solution was added slowly an equal volume of 94 per cent alcohol. The heavy flocculent precipitate was removed by centrifugation and dissolved in about 70 cc. of distilled water. This solution was acidified with N/1 acetic acid and the fine precipitate discarded. The solution was again precipitated with an equal volume of alcohol, and the precipitate redissolved in water. Precipitation and solution were repeated twice, the final solution, acidified with N/5 HCl, was dialyzed through cellophane until no Cl^- ions were demonstrable in the dialysate. The solution was then precipitated with ten volumes of acidulated acetone and the precipitate recovered by filtration. This product was labeled "C" B.

All "C" fractions gave strong positive Molisch reactions, and negative Millon and xanthoproteic reactions. We realize that the preparation of polysaccharide fractions of these organisms by the method which Avery and Goebel used to obtain an acetyl polysaccharide from the pneumococcus does not prove that our fractions "C" B are acetylated polysaccharides. At this time we merely report our findings with polysaccharide fractions of these organisms, obtained by two different methods. The "P" fractions gave positive Millon and xanthoproteic reactions, but also positive Molisch reactions, indicating that these fractions were not free from carbohydrate.

Equal quantities of carbohydrate fractions and diluted bacteriophages (0.03 cc. of each) were placed in small tubes and incubated for twenty-two hours at 35°C. A portion of each mixture was then overlayed on agar-plate susceptible cultures and later examined for plaques. None of the carbohydrate fractions "C" A, or "C" B, had any demonstrable inactivating effect, either on homologous or heterologous phages. These results are in accord with those published by Gough and Burnet (1934).

TABLE 8

	PLAQUE COUNT
Heat-killed staph. Vnt. with Bp. M-16	0
Heat-killed staph. v with Bp. M-16	0
Acid-treated staph. Vnt. with Bp. M-16	260
Acid-treated staph. v with Bp. M-16	260
Distilled water with Bp. M-16	260

That a carbohydrate associated with the somatic antigen may play some rôle in the ability of such antigens to absorb phages may be concluded from the following experiments. Agar-slant cultures of staphylococci were washed off with sterile distilled water. The resulting suspensions were then heat-killed by autoclaving. Each suspension was divided equally, one portion being retained as a heat-killed suspension, while the other was boiled in N/20 HCl for twenty minutes. The acid-treated suspension was then washed, resuspended in saline, and neutralized. To both the heat-killed and acid-treated suspensions equal amounts of staphylococcus bacteriophage were added and the mixtures incubated for eighteen hours at 35°C. Portions (0.02 cc.) were then tested by our usual technique for the presence of plaques. That heat-killed cultures that have been treated with acid lose their phage-inactivating effect may be observed from the results shown in table 8.

Protein fractions, "P" A, and "P" B, of enterococcus strains E-16, Z-1, Z-11, and E-9 and staphylococcus cultures "v," Vnt, and pg were tested for their phage-absorbing ability; 0.5 cc. of each fraction was added to 0.5 cc. of phage and the mixtures incubated for twenty-two hours at 35°C., when 0.02 cc. quantities of each mixture were plated for plaques against susceptible cultures. The results are listed in table 9.

TABLE 9

	PLAQUE COUNT
"P" A of culture E-16 with Bp. "Zy"	1
"P" A of culture Z-1 with Bp. "Zy"	0
"P" A of culture E-9 with Bp. "Zy"	0
"P" A of culture Z-11 with Bp. "Zy"	0
Saline with Bp. "Zy"	Over 200
"P" B of culture E-16 with Bp. "Zy"	2
Saline with Bp. "Zy"	311
"P" A of culture v with Bp. M-16	148
"P" A of culture Vnt. with Bp. M-16	0
"P" A of culture pg with Bp. M-16	0
Saline with Bp. M-16	161
"P" A of culture E-16 with Bp. M-16	87
"P" B of culture E-16 with Bp. M-16	134
Saline with Bp. M-16	312

The protein fractions from cultures of enterococci show as strong an ability to absorb homologous phage as do heat-killed and formalin-treated whole cultures. The protein fractions of culture E-16 also possess a significant amount of staphylococcus phage-inactivating action. Our results with the protein fraction of staphylococcus v run counter to those obtained with heat-killed cultures and watery extracts from autolyzed cultures. On three occasions we have prepared protein fractions of this culture and in each instance the fraction has had no phage-inactivating action toward staphylococcus phages M-16 and "V." We are unable, at this time, to give an adequate explanation for this exception. Protein fractions of other susceptible cultures of staphylococci behave with regard to their phage-inactivating action in a fashion similar to heat-killed cultures and extracts prepared from these strains.

DISCUSSION

The principle that bacteriophages, in general, are absorbed only by those strains of bacteria (heat-killed, or their extracts) that are visibly affected by contact with these lytic agents is well founded. Most of the facts, however, upon which this concept has been built have resulted from studies dealing with phages that attack the enteric group of bacteria. This large group of lytic agents is very slightly affected by such non-bacterial agents as pus, spinal fluid, whole blood, and serum. The staphylococcus bacteriophages are quite different. Most of them are weakened very considerably by contact (*in vitro*) with the same non-bacterial substances that ordinarily do not influence the action of phages which attack Gram-negative bacilli. They also differ in that they are absorbed by bacteria against which they have no demonstrable effect. Evidence has been obtained, however, to correlate the absorption of staphylococcus phages by organisms of the *Bacillus subtilis* group with the presence of staphylococcus-like antigen in these bacilli (Rakieten 1937).

The enterococci constitute another group of organisms which have a demonstrable inactivating effect on staphylococcus phages, in spite of the fact that contact with these lytic agents produces no observable changes in these microorganisms. On the basis of the results obtained with living cells, cultures killed by formalin and by grinding, we have concluded that enterococci have some antigenic component (surface?) which behaves like the surface antigenic component from susceptible staphylococci. That this component is distinctly heat-labile may be deduced from the fact that heated whole cultures, heated ground cultures, and watery extracts do not possess power to inactivate the staphylococcus phage. If this component is a surface one, then it must be present in very low concentration, since all of our attempts to produce precipitin which will react with staphylococcus precipitinogen have consistently failed. Since all of the rabbits we have employed had a high titer of naturally occurring agglutinin for staphylococci and a moderately high titer for enterococci, any increase in this antibody using heterologous antigen would be difficult to assess. No naturally occurring precipitin was found, and the production of this antibody by whole cells as well as by variously prepared protein fractions is still being attempted. If further study demonstrates that the staphylococcus-like component in enterococci is not detectable by ordinary serological methods, the value of phages for this purpose is very obvious.

The enterococci are unlike the three fixed types of pneumococci, studied by us, for we have been unable to demonstrate that these cocci consistently inactivate staphylococcus phages to any significant degree.

The results with the protein fractions of enterococci strengthen the view that these organisms have a fairly close relationship to staphylococci. Many more of these fractions are being studied, especially since fractions prepared from the same strains on different occasions differed in their phage-absorbing capacity.

Finally it becomes more and more evident why staphylococcus phages are so difficult to obtain from stool filtrates as well as from sewage filtrates. Apparently, diverse microorganisms are able to inactivate these lytic agents, making isolation uncertain from so-called rich sources of bacteriophage.

CONCLUSIONS

1. Actively growing cultures of enterococci possess a considerable power to inactivate staphylococcus bacteriophage.
2. Formalin-killed cultures as well as ground cultures also possess the same degree of activity.
3. Heat-killed whole cultures as well as heated ground culture material lose their ability to inhibit staphylococcus phages.
4. Watery extracts of enterococci absorb homologous phage, but have no demonstrable staphylococcus phage inactivating activity.
5. Staphylococci as well as some strains of *Bacillus subtilis* absorb an enterococcus phage.

6. Pneumococci, types I, II, and III, do not affect staphylococcus phages.

7. Carbohydrate fractions of enterococci are devoid of phage inactivating activity.

8. Protein fractions of these same strains will absorb homologous phage and to some extent heterologous phage (staphylococcus).

9. The component in enterococci responsible for staphylococcus phage inactivation can not be demonstrated, consistently, by the use of a serological method (production of precipitin).

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MICROBIOLOGY OF THE UPPER AIR

III. An Improved Apparatus and Technique for Upper Air Investigations[1]

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Received for publication February 23, 1938

Research in the microbiology of the upper air at the Massachusetts Institute of Technology was inaugurated in 1932. During the past five years, airplane collections of microorganisms and other air-borne materials have been made, in the course of more than two hundred separate flights in the vicinity of Boston. The first instrument used at the Massachusetts Institute of Technology was described by one of us (Proctor, 1934) together with certain of the findings made previous to that time.

Workers in this and other countries have been engaged in somewhat similar researches which have added a number of different devices capable of use for such collections, both from airplanes and balloons. Among the devices of more recent origin are those described by Lindbergh and Meier (1935), and Rogers and Meier (1936) in the United States, and Van Overeem (1936) in Holland.

The present paper is concerned with modifications in the bioaercollector and in the laboratory technique involved in its use which minimize the opportunity for contamination during collection and examination. The modifications also permit photographic records under aseptic conditions of the non-viable particulate matter which comprises the major portion of the collections, thus allowing subsequent culture of the living material collected.

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[1] Contribution No. 129 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

The collecting unit consists of a solid brass disc (plate 1, fig. 2) containing six holes in which collecting slides are held in small brass collars. Between these six holes are six smaller by-pass holes through which the air-stream flows, between collections. The collecting slides are circular 18 mm. no. 1 coverslips. A collar with a collecting slide in place is shown in plate 2, figure 6. The diameter of the collector holes is 20 mm., and the air-stream passes around the collecting slide through the 1 mm. annular space left when the collecting slide is in place. The brass disc is enclosed in a brass case (plate 1, figs. 1 and 3) and can be rotated by hand wheel (plate 1, fig. 1) to line up each of the collecting slides successively with the air intake. The case and disc have been carefully machined so that the flat surfaces fit tightly together. In addition, the contacting surfaces are covered with a heavy mineral oil. As a result of these precautions and the spatial arrangement of the collector holes, there appears to be no opportunity for contamination between collector holes or any chance of air flowing through more than one collector hole at a time. The air intake consists of a straight brass pipe with a double protecting collar which can be opened or closed by a sliding rod on the side of the collecting pipe. The air to be sampled enters through the top of the collecting pipe, passes down through the straight pipe, impinges directly upon the oiled collecting slide at right angles to the plane of the glass, flows around the slide through the annular space and out through the suction end of the collector. Air circulation through the device is obtained by a two-inch Venturi tube mounted on a strut of the plane and connecting with the collector by a rubber tube. The assembled apparatus is shown in plate 1, figure 4.

The equipment is prepared for use in the following manner: The disc and the inside walls of the enclosing case are cleaned and oiled with a heavy, clear, colorless mineral oil. Cover slips cleaned and previously stored in 95 per cent alcohol are flamed and placed in the holders which are then fixed firmly in the disc. Filtered mineral oil is placed upon these cover slips on the side facing the air intake, thereby constituting an adhesive surface upon which the air impinges. The retained material is the source of our collections. The upper surface of the cover slip is nearly 1 mm. below the top of the collector hole so that oil from the case will not be drawn by capillarity on to the slide and thus contaminate the specially cleaned oil on the collecting slide. The apparatus is then assembled as a unit and sterilized in a hot air oven at 180°C. for two hours.

The collector is mounted in the cabin or cockpit of the plane by means of a circular clamp, so that the collector pipe extends outward into the slipstream of the propeller. The protective cap prevents dust from entering the collector during flight preparations and take-off. At an elevation of 1500 feet the protective cap is opened and a ten-minute sample taken by allowing air to be drawn in over the oiled cover slide.[2] The brass disc is turned to the next hole which is a by-pass opening and left for a period of one minute, after which the disc is again turned to the succeeding collector hole where another collection is made in the same manner as before. Following this same routine the remaining slides are exposed, with an interval between each exposure of a one-minute flow of air through each successive by-pass hole, thus permitting an air wash. At the end of the fifth exposure, the tightly fitting protective cap at the exterior end of the collecting pipe is manually closed from within the plane.

As stated previously, there are six collecting slides (cover slips) mounted in the disc. The sixth slide is not exposed; it serves as a control, and the device is so arranged that this slide cannot be lined up with the air intake. When examined with the other slides, this control slide serves as a reliable index of imperfect sterilization or contamination.

During the past two years our equipment has been flown and operated by army pilots in the Boston weather plane. These flights were made to an altitude of 16,500 feet as the maximum elevation. On this schedule five samples were taken from 1,500 feet to 16,500 feet; each sample being taken during a 3,000 foot climb.[3]

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[2] The rate of air flow through the instrument is approximately 1.1 cubic feet per minute and was determined by laboratory calibration of the pressure differential at average flight speeds. A larger instrument with a greater air flow and more numerous collecting slides is now under construction.

Examination of the collected material is carried out in the following manner: The inlet side of the case (plate 1, fig. 3) is removed and the exposed side of the disc is quickly placed upon a brass plate (plate 2, fig. 5) on which three sterile microscope slides are arranged. The oil on the disc causes the microscope slides to adhere and cover the six collector holes when the disc is lifted. The other half of the case is then removed, the disc is inverted and placed upon a similar brass plate (plate 2, fig. 7). The holes on the other side are covered in the same manner by protective microscope slides. As a result the collector holes on both sides of the disc are protected against contamination.

The disc, containing protected collecting slides, is placed upon the pedestal plate (plate 2, fig. 8) which is then mounted in a modified mechanical stage of a microscope. The disc may be rotated on this plate so that the protected collector slides may be examined and photographed. Representative areas are then selected and photographed on 35 mm. motion picture film, thereby yielding permanent records. Calibrations of the microscope and camera permit quantitative comparison of the collected material.

After examination and photography are completed the protective slides on the under side of the disc are removed. Each upper protective slide is moved sidewise to expose one hole at a time and the corresponding collecting slide holder is moved upward to allow removal of the collecting slides. Each collecting slide is transferred by sterile forceps to a sterilized four-ounce rubber-stoppered bottle which contains 7 m.l. of sterile distilled water. After the bottles have been shaken, melted standard nutrient agar[4] of double concentration is added and mixed. The bottles are slanted and incubated at 20°C. and 37°C. for several days at each temperature, after which the organisms are subcultured and identified.

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[3] Previous to this coöperative arrangement between the U. S. Army Air Corps and the U. S. Weather Bureau, collections were made during the course of flights to heights over 20,000 feet by a meteorological plane owned and operated by the Massachusetts Institute of Technology. The results of these flights were reported in the Journal of Bacteriology (Proctor, 1935).

[4] Other specialized types of culture media have been used for the development of particular types of microörganisms.

If any microorganisms develop from similar treatment of slide 6, the control, the collections of the entire flight are rejected. The heat sterilization of the intact collector previous to use and the oil seal between disc and case, combined with the closure mechanism of the inlet orifice, appear to prevent contamination. Only in rare instances has any growth been found from the control slide.

The possibility of dust or organisms being temporarily retained in the collector pipe and dislodged to contaminate a succeeding collection has been minimized by covering the inner surfaces of the collecting pipe and the protective cap assembly with the same heavy mineral oil used in the collector itself. Particles striking these oiled surfaces adhere in the same way as they do on the collector slides. From an aerodynamic viewpoint, the air velocity close to these surfaces is much lower than that in the major internal portion of air stream. Therefore it is considered that the possibility of dislodging these trapped particles is very slight.

As the inlet end of the collector pipe is in the slip-stream of the propeller, there is a possibility that materials originating on the motor housing or the propeller assembly could enter the collector. To minimize this type of contamination, the oiled protective cap is kept closed during the time preparatory to take-off and until the plane has reached an elevation of 1500 feet. Air flow tests indicate a relatively high velocity close to these surfaces. This results in a substantial air wash of nearly thirty minutes previous to the time the collector is opened, which would seem to be a sufficient interval to dislodge from the smooth, highly polished surfaces of the plane most materials which might later influence the collections. The fact that in numerous instances we have found no viable organisms in an entire collection would seem to preclude the possibility of gross contamination from such surfaces. Until such time as our equipment may be mounted anterior to the propellers, this possibility of contamination cannot be entirely excluded.

Since the start of this work, a large number of bacteria and molds have been collected and identified. Using the same improved apparatus, just described, forty flights were made over greater Boston from November, 1936 through June, 1937.

TABLE 1

HEIGHT	BACTERIA*	MOLDS†
feet		
1,500–4,500	Achromobacter rathonis Bacillus cereus	Aspergillus fumigatus Aspergillus niger Aspergillus flavus Macrosporium sp. Penicillium frequentans
4,500–7,500	Bacillus ruminatus Bacillus aerosporus Bacillus simplex Bacillus albolactis	Aspergillus niger Aspergillus glaucus Cladosporium sp.
7,500–10,500	Sarcina lutea Bacillus megatherium Bacillus prausnitzii Bacillus albolactis	Aspergillus niger Hormodendrum herbarum
10,500–13,500	Bacillus cereus Kurthia zopfii	Aspergillus calyptratus Hormodendrum herbarum
13,500–16,500	Micrococcus candidus Bacillus subtilis Bacillus albolactis Bacillus simplex	Penicillium glabrum Penicillium lanosum

* Bergey's Manual, edition 4, was used for identification.

† The authors are indebted to Dr. David H. Linder of the Farlow Herbarium, Harvard University, for assistance in the identification of certain of the molds.

Photographic records have been made of the particulate material collected during these and some earlier flights. Of the organisms obtained in the latest series, sixteen are bacteria and nineteen are molds.

As these collections are made during flights which simultaneously procure air mass data, the possibility of correlating the presence of any particular micro-organism with different types of air masses is apparent.

In table 1 the micro-organisms collected and identified in whole or in part are cited, together with the altitude ranges at which they were collected.

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PLATE 1

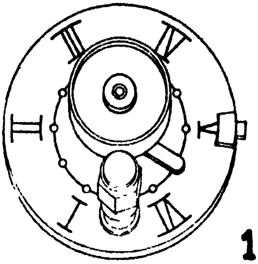
FIG. 1. The lower half of the enclosing case showing the centrally-located hand wheel by which the disc is turned. Between numerals *I* and *V* is the covered suction outlet. The cap is kept on until the collector is installed in the plane. The case is held together by an extension of the central shaft of the hand wheel. This shaft is not visible on this drawing.

FIG. 2. The collecting disc showing the six large collector holes in which the oiled collecting slides are mounted. Between these holes are the six smaller by-pass holes.

FIG. 3. The upper half of the enclosing case. The inlet hole is barely visible in the lower portion of the drawing.

FIG. 4. The assembled apparatus with the collecting orifice opened.

PLATE I



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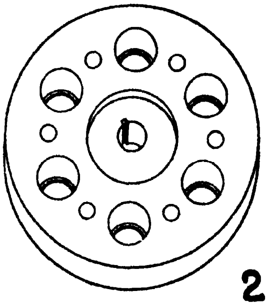

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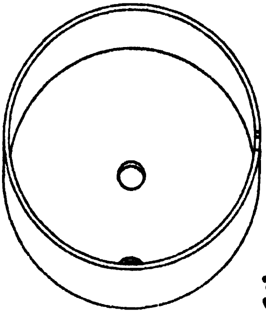

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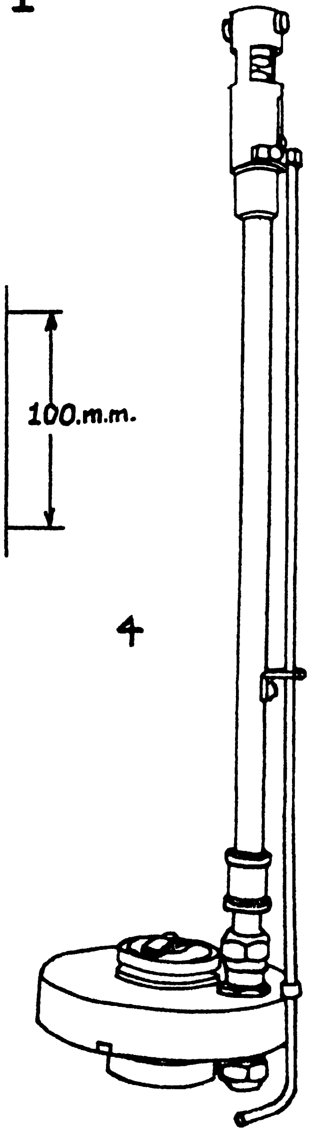

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PLATE 2

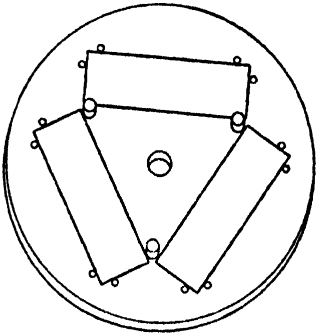
Figs. 5 and 7. Brass Plates for applying protective microscope slides to the collector disc preparatory to laboratory examination and photography.

Fig. 6. Enlarged view of a collecting slide and slide holder with which each of the six large holes in the disc (plate 1, fig. 2) is fitted.

Fig. 8. Pedestal plate upon which the protected disc is mounted for examination.

Figs. 5, 7 and 8 are drawn to the same scale as the figures in plate 1.

PLATE II



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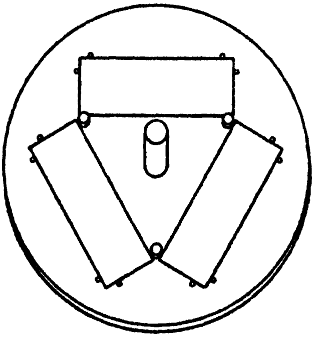

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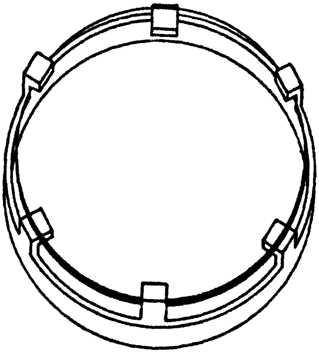

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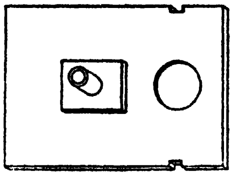

8

COLCHICINE STIMULATION OF YEAST GROWTH FAILS TO REVEAL MITOSIS

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Received for publication February 28, 1938

The formation of the yeast bud has generally been believed to be a partitioning of the materials between the mother cell and the bud, rather than involving a mitotic division of the nuclear material. Three investigators have presented evidence for a mitotic division, but the evidence has not been entirely convincing. Colchicine delays mitotic division at the metaphase and has been helpful in the study of cell division in both animal and plant cells. A proper concentration of the drug should delay any mitotic division of yeast, change the rate of growth and permit the observation of division figures. Instead, colchicine stimulates yeast growth and fails to show mitosis.

I

The yeast used is a pure strain of *Saccharomyces cerevisiae* Hansen from a single cell isolated by the writer in 1928 (A. T. C. No. 4360) and was grown at 28°C. in 10 ml. Williams' original medium consisting of sugar 20 grams, $(NH_4)_2SO_4$ 3 grams, KH_2PO_4 2 grams, asparagin 1.5 grams, $CaCl_2$ and $MgSO_4$ each 0.25 gram aq. dest. 1000 ml. The medium was tubed, sterilized and inoculated as described by Richards (1932). Mallinckrodt's U. S. P. colchicine was used in the experiment in preference to Merck's colchicine as the latter was not chloroform free. Two check experiments with the Merck drug gave very nearly the same results so that with yeast either might have been used and without significant differences.

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[1] Experiments series 162 and 163 were grown at New Haven, 164 at Woods Hole and 155 and 156 at Buffalo.

The growth of the yeast was measured by counting the number of cells present in 1/250th cu. mm. with an hemocytometer; by means of a photoelectric nephelometer (Richards and Jahn, 1933), improved by using a General Electric Blocking Layer Photoelectric Cell and a Weston Model 600 microammeter with knife edge pointer and mirror; and by centrifuging the cells into calibrated, capillary bottom, centrifuge tubes. More than one criterion of growth is necessary for the analysis of the conditions determining growth (Richards, 1934) and the interrelations of the several criteria will be discussed elsewhere. The metal surface of the Bright Line Hemocytometer permits more rapid filling of the cell by capillarity, thereby lessening the error due to unequal distribution of the yeast. The growth measured by the nephelometer is a function of the number of cells present and their size distribution and is expressed as

$$E = \log I - \log I_0$$

when I is the density of the suspension at the time of measurement and I_0 the density of the suspension at the time of seeding the population.

II

Concentrations of colchicine weighed out and added in the proportion of one to one million parts of medium gave very little less growth than the control populations and concentrations greater than ten times this amount stimulated the yeast growth progressively until a concentration of 1 per cent was reached. Four and one-half per cent concentration, which is near the solubility limit of the drug, gave less stimulation (fig. 1, *A*). In terms of percentage of the control the amounts of stimulation of cell number and nephelometric density were about the same and the agreement of the several determinations is shown by the figure. The average size of the yeast cells grown in the medium and in the medium plus 1 per cent colchicine and the distribution of the sizes of the cells showed no significant differences; as determined by photographing the yeast, projecting the images of the photographs, tracing them and measuring the area of the

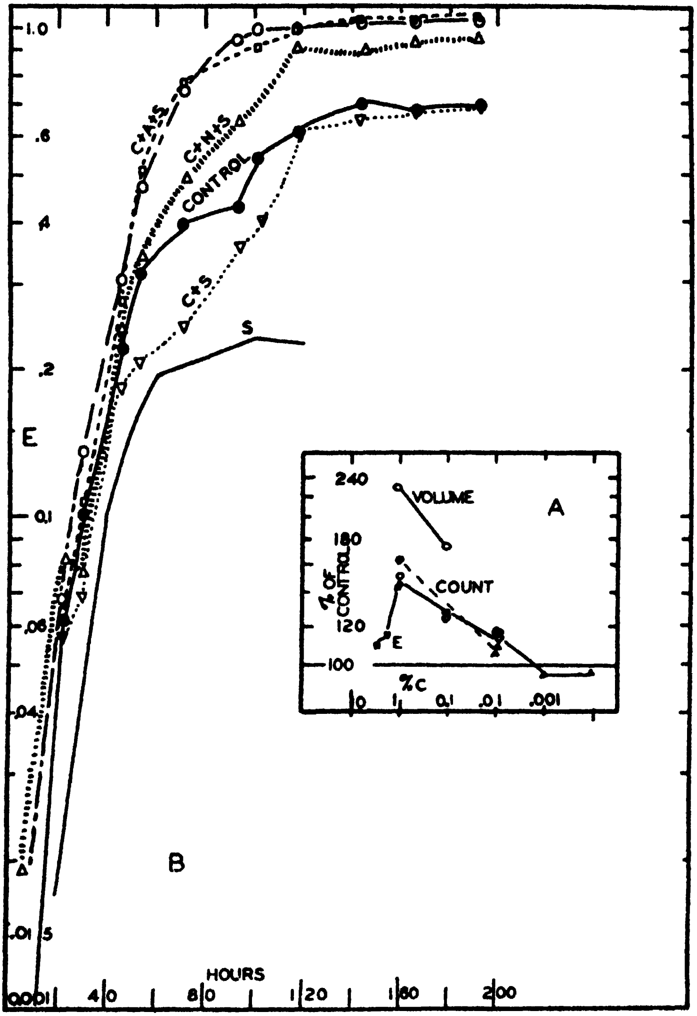

Fig. 1. *A*, the Increased Yield of Yeast with Added Colchicine. *B*, the Growth of Yeast Populations in Different Combinations of Nutrients (*cf.* Text)

Control in Williams' medium. *C* = colchicine. *S* = sugar. *A* = asparagin. *N* = $(NH_4)_2SO_4$.

enlarged photographs with a planimeter. Consequently, the proportionally greater volume must be due to inefficient packing of the cells into the capillary tubes by the centrifuge.

The increased yield of yeast, while marked, is not of the order of magnitude to be expected from the presence of a bios, because far greater concentrations of the drug are required for a comparable stimulation. To determine the nature of the stimulation, series 164 was analysed to learn the changes in the sugar, alcohol, and hydrogen ion concentrations at frequent intervals during the growth cycle. Previous experience had shown that the sugar concentration is a measure of the food in the medium used by the cells and that the unfavorableness of the medium from the accumulation of waste products was proportional to the fermentation which could be measured in terms of the alcohol concentration (Richards, 1932).

The growth of the cells in the medium without the drug produced two cycles (fig. 2), which are characteristic for this yeast in this medium. The growth curve of the yeast grown in 1 per cent colchicine in addition to the medium grew directly to a maximum crop in a single cycle of growth. The concentration of sugar in the medium is about the same during the first 60 hours and then decreases more rapidly than in the control cultures. The hydrogen ion concentration is diminished when the drug is added, but that of the colchicine cultures then increases faster than in the control cultures. The alcohol concentration in the colchicine cultures increases to a maximum some six times greater than that of the control cultures and does not show a period of equilibrium associated with the end of the first cycle of growth such as is observed in the alcohol concentration of the control series.

The equilibrium marking the end of the first growth cycle of the control cultures results from a selective killing of the larger buds by the toxic waste products in the medium and a decrease in the available food supply. The addition of food just before the end of the first cycle prevents the retarded growth and the population continues directly to a maximum crop limited by the medium becoming too unfavorable for further increase (Richards, 1934).

As only one cycle occurs when the colchicine is present, the addition of the drug must act either as a food or by making the medium less unfavorable even though the alcohol and other products of fermentation are proportionally greater. To evaluate the extent of these two possibilities an experimental series was grown in the complete medium, the medium with 1 per cent colchicine, and in 1 per cent colchicine with the mineral salts and the following combinations of nutrients: sugar, ammonium sulfate, asparagin, asparagin and ammonium sulfate, asparagin and sugar, and ammonium sulfate and sugar, the concentrations being

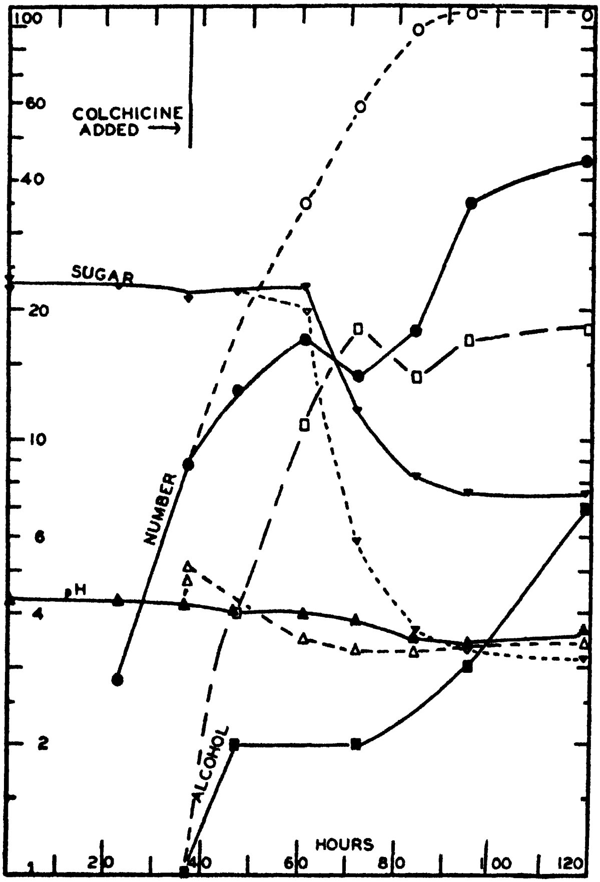

Fig. 2. Increased Number of Yeast Produced by the Addition of Colchicine and the Changes with Time of the Sugar, Hydrogen Ion and Alcohol Concentrations of the Medium with and without the Drug

exactly the same as in the control medium except for the nutrients intentionally omitted.

Without sugar, little growth was observed. With the sugar and the mineral salts an appreciable growth occurred, (fig. 1, *B*). This curve was added from a previous series started with a smaller seeding which accounts for the curve being to the right of the others. The similarity of the slopes, which show the relative rate of growth from the plotting on arithlog paper, show that the rate was essentially the same except for less yield. The addition of 1 per cent colchicine to the sugar and mineral salts increases the yield nearly three times and there is a suggestion of two cycles in the growth curve. The crop attained is the same as that of the control series without the drug but in the complete medium. The yeast may obtain nitrogen from the colchicine, although not as readily as from the asparagine and ammonium sulfate.

The addition of ammonium sulfate to the colchicine and sugar gives a still greater yield, but the period of decreasing rate of growth occurs earlier than when the drug is added to the complete medium. The colchicine, asparagin and sugar gives a crop similar to the complete medium, so that the colchicine serves as well without the ammonium sulfate. Asparagin and colchicine without sugar give less yield than with sugar alone and this curve was not plotted.

The experiments show that the colchicine is a food and also buffers the medium, lessening the effect of the increasingly adverse medium on the yeast proliferation so that the population grows directly to the maximum crop without passing through two growth cycles. The period of constant relative rate, or logarithmic growth, lasts longer when the drug is present with an additional source of nitrogen, preferably asparagin, despite the fact that the alcoholic fermentation is greater and the sugar is used up more rapidly.

III

Had the colchicine arrested the division, or bud formation of the yeast as it does with other plants and animals (Nebel, 1937; Allen, 1937) the rate of growth of the populations would have been less than of the control populations grown without the drug. Even a small retardation would be evident during the period of exponential rate of incrase and would appear as a changed slope of the growth curves. The logarithmic growth period continues beyond that of the control cultures when the colchicine is added to the culture medium and the departure from the straight line in the figures plotted on arithlog paper occurs later and less rapidly. The slightly lesser crops obtained with the lower concentrations of the drug differ from the controls by less than the experimental error (4 per cent) and the differences are not significant (fig. 1, *A*).

Samples of yeast were prepared for microscopic examination by killing in Lavdovsky's or Bouins fluids, mordanted in 2.5 per cent iron alum, stained in 1 per cent aqueous hematoxylin, destained in iron alum or in picric acid and mounted with glycerine jelly. Some of the preparations were made in bulk and the cells separated from the various fluids by gentle centrifuging and the rest as cover glass preparations.

None of the stained preparations showed any grouping of the chromatin that might resemble chromosomes. The chromatin granules were irregularly distributed between the mother cell and the bud depending somewhat on the size of the bud. I failed to note any distinguishing difference between the appearance of the colchicine-treated and the control cells. The yeast were examined at different stages during the growth cycle.

Wager (1898), Wager and Peniston (1910) and Guilliermond (1904–1912), among others, failed to find mitosis in their extensive studies. Fuhrman (1906), Swellengreble (1905) and Kater (1927) describe a mitosis of the yeast nucleus with four chromosomes. The first mentioned paper seems not to be entirely clear. The other two papers publish clear descriptions and figures. Unfortunately the preparations of Kater have faded and are not available for examination.

No yeast cell that I have seen has shown any grouping of chromatin that might be interpreted as chromosomes. Feulgen-stained preparations have been studied (1933). This includes examination of my own preparations and some preparations very carefully stained by Dr. H. W. Beams for this purpose. The ultra-violet photographs of Ter Louw and of Dr. F. F. Lucas do not show chromosomes (personal communications to the author). The preponderance of negative evidence does not answer the question in a satisfying manner, but it is peculiar that only three observers have been able to see mitotic figures if the budding of yeast is mitotic. It was hoped that the colchicine would make possible the demonstration of mitotic division as it has done with such success for other cells. The drug failed to show any effect on cell division although it does stimulate the growth of populations of yeast.

SUMMARY

The colchicine technique was applied to the study of the growth of yeast populations and failed to show any evidence of other than amitotic cell division. Stained preparations of the cells grown in Williams' medium and in the medium plus from 4.5 per cent to 1 part of colchicine to ten million parts of medium showed no differences in cytological structure. One per cent of the drug gave maximum stimulation of the yeast growth. The stimulation is not of the kind given by a bios, but is due rather to the colchicine serving as a food and as a buffer in lessening the adverse effect of the increasingly unfavorable medium on the growth of the yeast populations.

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A SIMPLE METHOD FOR THE STERILE FILTRATION OF SMALL AMOUNTS OF FLUID

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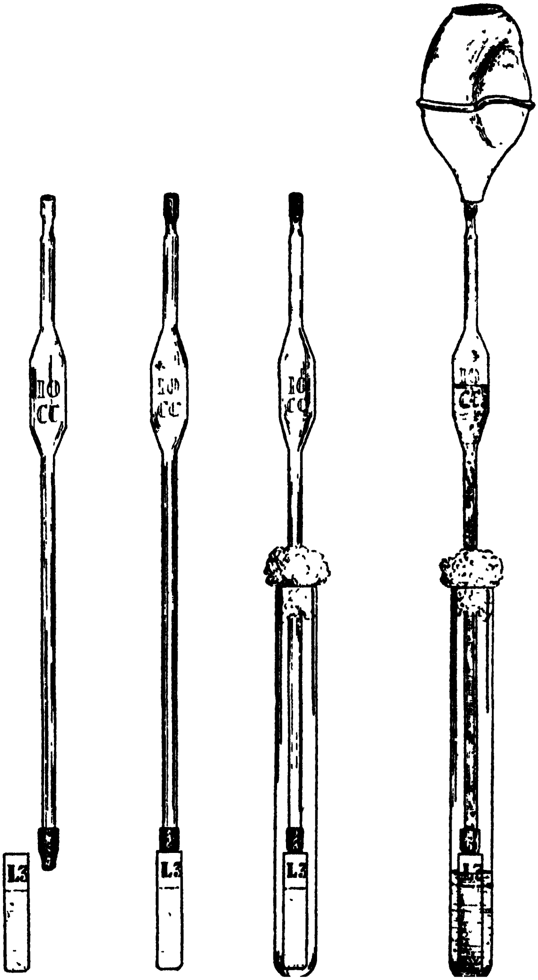
Received for publication March 5, 1938

In many procedures associated with the study of bacteriophage the filtration of relatively large amounts of fluid is seldom necessary, a few drops of the filtrate usually being quite sufficient for trial purposes. The use of Berkefeld, Mandler, Seitz or similar filters requires careful preparation of apparatus and even the comparatively simple use of Chamberland candles or Collodion membranes described elsewhere (1933) demands considerable time and attention.

After numerous attempts to devise a method whereby a few cubic centimeters of sterile filtrate could be quickly and easily obtained, the following procedure was found to be entirely satisfactory:

A miniature candle (55 x 10 mm.) of the Chamberland L_3 or L_5 series is employed. The latter is fitted to the tapered end of a 10 or 20 cc. transfer pipette (student grade) by means of a short piece of thin tubing (3 mm. bore, 2 mm. wall) of pure gum rubber. To prevent leakage between the candle and tubing the connection is sealed with a watery emulsion of latex (marketed under various names as adhesive and also sold as a substitute for leather in the repairing of shoes). The upper end of the pipette is shortened to a point about 3 inches from the bulb and a slight constriction is made about 1 inch from the end to prevent a cotton wool plug from slipping down the pipette. The mounted candle is then placed in a long test tube and sterilized in the autoclave.

When required for use, the candle is immersed in the fluid to be filtered approximately half way to the glazed neck. Suction is applied to the end of the pipette either by fitting over it a compressed india-rubber bulb or preferably by connecting it to a vacuum pump. When the latter is employed, about 10 cc. of sterile filtrate can be obtained in less than five minutes, even with heavy bacterial suspensions.



After sufficient filtrate has appeared in the bulb of the pipette, the latter is disconnected from the pump, the cotton plug removed, the end flamed and the filtrate withdrawn with a Pasteur pipette. The entire process requires only five or ten minutes and a large number of test filtrates can be obtained in a short time.

To keep the candles in a satisfactory condition for prolonged and repeated use, the following simple procedure is recommended:

Using the same Pasteur pipette that was employed for collecting the filtrate, fill the volumetric pipette with distilled water and allow the latter to flow back through the candle by gravity, thus removing any material adhering to the outside wall of the candle. After sterilization in the autoclave, the candle is disconnected from the pipette, dried and burned in a muffle furnace at dull red heat for 1½ hours. Filter candles thus treated may be used repeatedly and will remain in a satisfactory condition for an indefinite period.

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NUTRITION OF THE PROPIONIC ACID BACTERIA[1]

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Received for publication March 11, 1938

Wood, Tatum and Peterson (1937) and Tatum, Wood and Peterson (1936a) have shown that an ether-extractable acidic factor found in aqueous extract of yeast is essential for growth and fermentation of propionic acid bacteria in a synthetic medium containing ammonium sulfate as the source of nitrogen. Although growth was obtained with this combination, the organisms generally failed to multiply after the third or fourth transfer. The addition of vitamin B_1 and hydrolyzed casein made repeated transfer possible with consistent results. Vitamin B_1 stimulated growth, particularly in the presence of amino acids. Certain propionic acid bacteria were found to grow with equal vigor with or without the addition of vitamin B_1 and may be able to synthesize this vitamin.

These investigations indicated that amino acids were essential for vigorous and continuous growth in the supplemented synthetic medium. The present study was made to determine which of the amino acids are important in the general nutrition of propionic acid bacteria. It has been found that amino acids are not essential for growth.

EXPERIMENTAL

Cultures

Each of the seven species used was checked for purity by fermentation of the differential sugars in the classification of Werkman and Brown (1933). The following cultures were used:

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[1] Journal Paper No. J-540 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 572. Supported in part by Industrial Science Research Funds of Iowa State College.

Propionibacterium rubrum 9W; *P. peterssonii* 11W; *P. thönii* 23W, 39W; *P. zeae* 35W; *P. pentosaceum* 49W; *P. shermanii* 52W; and *P. freudenreichii* 53W. Cultures 35W, 49W and 53W are transplants of cultures 56, 11 and 33 respectively, as referred to in the previous studies by Tatum *et al.* (1936a) and Wood *et al.* (1937).

Medium and analytical methods

The media developed in previous work were used throughout this investigation. The basal medium contained: glucose, 1 per cent; sodium acetate, 0.6 per cent; ammonium sulfate, 0.3 per cent; and Speakman's salts in half concentration. The growth stimulants were added in various combinations and in the following concentrations: ether extract of 0.3 gram of Difco yeast extract; 0.1 gamma of crystalline vitamin B_1 (Merck's); 10 gamma of crystalline riboflavin (Borden) per 10 cc. of medium, unless the concentration is otherwise designated. The amino acids were used in a concentration of 0.02 per cent on the basis of the naturally occurring isomer with the exception of cystine and tyrosine which were used in a concentration of 0.01 per cent. The following amino acids were used: glycine, dl-alanine, dl-valine, dl-isoleucine, dl-leucine, dl-phenylalanine, l-tyrosine, l-cystine, dl-methionine, l-tryptophane, l-proline, l-hydroxyproline, l-aspartic acid, d-glutamic acid, d-arginine, dl-lysine and l-histidine. Hydrolyzed casein was used in 0.15 per cent concentration. Ammonium sulfate was added to all media containing amino acids, as it was our purpose to determine the influence of amino acids in the presence of ammonium sulfate. The constituents of the media were adjusted to pH 7.0, combined, diluted to volume with distilled water, and sterilized at 15 pounds pressure for 20 minutes. Five cubic centimeters of medium were used in test tubes 1 cm. in diameter. Incubation was aerobic at 30°C. for 4 or 5 days. The inocula obtained from yeast extract medium were centrifuged and resuspended in an equivalent volume of water; cells grown in supplemented synthetic medium with ammonium sulfate as a nitrogen source were not centrifuged. One drop of inoculum was used per tube unless otherwise stated. Fermentation was followed by direct titration of the acid formed, with bromothymol blue as an indicator.

Amino acid requirements

The results (table 1) show clearly that amino acids have an important function. Four cultures having no amino acids gave only slight growth and no acid on the second transfer; on the third transfer there was no visible growth in four days. Culture 35W grew more vigorously than the other cultures; it was not until the fifth transfer that all visible growth disappeared. The addition of amino acids stimulated growth of each of the cultures. There was a luxuriant development of cells and consistent acid production throughout five transfers. Implications at this time were that one or more of the seventeen amino acids was essential and that growth was not possible with ammonium sulfate after material included in the inoculum was depleted by serial transfer. In an attempt to determine which were necessary, the seventeen purified amino acids were separated into the six groups shown in table 2. Two cultures were used in the test. Experiments with culture 49W were run independently; transfers were made with culture 52W from the first set of tubes (c) to a second set (d) in which the amino acids were one-half as concentrated. The first tests were made by eliminating singly each of the six groups of amino acids to determine whether any one group contained essential amino acids (numbers 1 to 7). No single group of amino acids was essential. With culture 49W the elimination of groups IV and V (numbers 5 and 6) caused no decrease in growth compared with a medium containing all six groups (number 1), indicating that groups I, II, III and VI were the more important for this culture. Culture 52W showed some decrease in growth on elimination of each single group from the six, but groups I, II and III seemed the more important, particularly in series (d). The combination of groups I, II, III and VI were tested next (number 8). Culture 49W grew practically as well as with the complete set of amino acids (number 1) or hydrolyzed casein (number 25). Culture 52W did not give as good growth on this combination as on the seventeen amino acids or hydrolyzed casein but again (in series (d)) groups I, II and III (numbers 9, 10, 11) seemed important. Growth was retarded by the removal of any one of these groups. Where two groups were used (numbers 13 to 17) the growth was usually somewhat weaker than with three, and with a single group growth was retarded even more. Cystine (number 23) seemed to have a favorable influence but growth on a second transfer from this tube was weak. It is apparent that clear cut differentiation of the important amino acids is not possible, at least under the conditions of these experiments. Possibly by direct determination of cell weight with a short incubation period, the more important amino acids could be ascertained. However, it seemed that uncontrolled factors other than the methods of analysis were influencing our results. Subsequent data in this paper show that these cultures readily acquire the ability to synthesize amino acids, and can utilize ammonium sulfate as the source of nitrogen if properly "trained" in the sense of Knight (1936) who defines the term as "the derivation of cultures having simple nutrient

TABLE 1

Comparison of ammonium sulfate and purified amino acids as a source of nitrogen for propionic acid bacteria*

Medium contained B_1 and ether extract of yeast extract

NUMBER OF TRANSFERS	CULTURE 11W		CULTURE 35W		CULTURE 39W		CULTURE 52W		CULTURE 53W	
	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added
	Cubic centimeters of 0.1 N acid per 10 cc. of medium									
1	1.6	2.1	3.3	4.0	0.2	2.9	1.1	4.2	1.1	4.0
2	0.0	3.0	1.7	4.1	0.0	2.6	0 0	5.1	0.0	3.8
3		2.6	1.2	3.5		2.8		4.5		3.3
4		2.7	0.0	4.5		2.5		5.1		4.3
5		3.1		4.7		3.0		4.5		4.1

4-day incubation. Initial inoculum was obtained from yeast extract glucose medium.

* Mixture of seventeen amino acids, 0.02 per cent of each except cystine and tyrosine 0.01 per cent, calculated on the basis of the natural isomer.

TABLE 2

Comparison of amino acid mixtures as a source of nitrogen for the propionic acid bacteria

Medium contained ether extract of yeast extract and B₁

NUMBER.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
I. Glutamic, leucine, histidine, methionine		+		+	+	+	+	+	+		+	+	+	+		+			+							Hydrolyzed casein
II. Glycine, alanine, valine		+	+		+	+	+	+	+	+		+	+	+	+		+			+						
III. Isoleucine, aspartic, lysine, arginine		+	+	+		+	+	+	+	+	+		+		+	+		+			+					
IV. Proline, hydroxproline, phenylalanine, tryptophane		+	+	+	+		+	+														+				
V. Tyrosine		+	+	+	+	+		+															+			
VI. Cystine		+	+	+	+	+	+		+	+	+	+					+	+						+		
Number of groups present		6	5						4	3				2					1						0	
cc. of 0.1 N acid produced per 10 cc. of medium	Culture 49W a*	5.0	4.1	4.8	4 1	5.1	5.3	3.7	4 9		4.8	3.2	4.5	3.6											2.0	5.0
	Culture 49W b*	4 8		4.3	3.7	4.8	4.9		4.6		4.6					3.4		4.0							1.8	5.0
	Culture 52W c*	4.9	4.5	3.7	4.0	4.4	4.5	4.4	4 6	3.7	3.1	3.1	2.6		3.2	3.2	2.0	2.8							0.3	4.6
	Culture 52W d*	4.0	3.4	3.0	2.7	3.6	3.6	3.5	3 6	2 4	2 5	2.7	3.6		2.6	2.1	1.8	2.4	0.7	1.9	1.5	0.5	0.3	2 8	0.4	4.0

4-day incubation.

* a, b and c tubes contained 0.02 per cent of each of the amino acids tested except tyrosine and cystine (0.01 per cent); d tubes contained 0.01 per cent each of the amino acids tested except tyrosine and cystine (0.005 per cent). Inoculum for a and b tubes obtained from supplemented synthetic medium. c tubes' inoculum obtained from yeast extract glucose medium. d tubes inoculated from corresponding c tubes except number 18 through 23 were inoculated with number 24.

requirements from cultures with a complex nutrition." It seems probable that in the presence of a few amino acids the ability to dispense with other amino acids is acquired readily. This ability may account for the lack of specificity for any particular amino acid as illustrated in table 2. Gladstone (1937) has made similar observations with *Staphylococcus* showing that "training" to synthesize one amino acid may enhance the ability to synthesize other amino acids. The poor growth on ammonium sulfate medium may result from the difficulty of "training" in this medium. Apparently, in the absence of all amino acids the bacteria only slowly acquire an ability to synthesize those amino acids that are essential. The differences in growth noted in table 2 on the elimination of certain groups of amino acids are probably a measure of the difficulty of synthesizing or dispensing with, a certain amino acid.

Stimulation of growth by riboflavin

Wood, Andersen and Werkman (1937) reported the stimulating effect of riboflavin on propionic acid bacteria. Further results are presented in tables 3, 4 and 6. Table 3 shows the acid production during serial transfer of three cultures in ammonium sulfate medium, with and without the addition of riboflavin. It is apparent that riboflavin had a stimulating effect on the growth of each culture. The growth of 35W and 49W was consistently vigorous throughout six transfers when flavin was added but decreased and was very weak at the conclusion of the experiment in its absence. Culture 52W failed to grow after the fourth transfer. The stimulating influence of the flavin was evident with this culture particularly in the early stages of growth. It served to shorten the lag phase and turbidity was evident much earlier. Cultures 9W, 11W, 23W, 39W and 53W failed to grow in either medium. It was necessary to "train" these cultures in order to obtain even moderate growth in ammonium sulfate medium containing flavin. These organisms apparently do not acquire the ability to synthesize amino acids as readily as others. On the other hand they are able to synthesize or dispense with flavin in amino acid medium for this substance was not necessary for vigorous growth in the presence of amino acids (table 1). The effect of different concentrations of riboflavin on acid production by propionic acid bacteria in ammonium sulfate medium is shown in table 4. Five-tenths gamma of riboflavin per 10 cc. had a distinct effect. The maximum influence was reached at 10 gamma per 10 cc. Snell, Strong and Peterson (1937) observed a stimulation of lactic acid bacteria by 0.05 gamma per 10 cc. and a maximum at 0.5

TABLE 3

Effect of riboflavin on acid production by propionic acid bacteria

Medium contained ether extract of yeast extract, B_1 and no amino acids

CULTURE*	FLAVIN ADDED TO 10 cc. OF MEDIUM	NUMBER OF TRANSFERS					
		1	2	3	4	5	6
		Cubic centimeters of 0.1 N acid per 10 cc. of medium					
	gamma						
35W	None	2.3	2.0	2.4	0.9	1.2	0.5
	10	3.7	3.5	4.5	3.2	4.0	4.2
49W	None	3.1	2.2	2.8	1.4	1.0	0.2
	10	3.6	3.6	4.4	3.6	4.0	3.7
52W	None	3.3	2.7	3.1	0.1	0.0	
	10	3.6	3.6	4.0	0.6	0.0	

5-day incubation. Initial inoculum was obtained from yeast extract medium.

* Cultures 9W, 11W, 23W, 39W, 53W failed to grow in this experiment.

TABLE 4

Effect of concentration of riboflavin on acid production by propionic acid bacteria

Medium contained ether extract of yeast extract, B_1 and no amino acids

CULTURE NUMBER	GAMMA OF FLAVIN ADDED PER 10 CC. OF MEDIUM				
	None	0.5	5.0	10.0	20.0
	Cubic centimeters of 0.1 N acid per 10 cc. of medium				
35W	1.3	2.6	3.2	4.0	4.1
49W	2.2	2.7	3.6	4.4	4.4
52W	2.9	3.5	3.6	3.6	4.1

5-day incubation. Inoculum was obtained from second transfer on the supplemented synthetic medium containing no flavin or amino acids.

gamma per 10 cc. Orla-Jensen, Otte and Snog-Kjaer (1936) first observed the stimulating effect of riboflavin on lactic acid bacteria. Lava, Ross and Blanchard (1936) suggested that flavin or B_2 might be a stimulant for propionic acid bacteria but were unable to prove this because of the impurity of their preparation. They fractionated yeast extract and found the fraction that contained B_2 was the most active. In the present investigation crystalline riboflavin was used and the effect is definitely shown to be produced by the flavin.

"Training" and the effect of size of inoculum

The same medium was inoculated with 1, 2 and 5 drops of the suspension per 5 cc. The cells for the inocula were obtained by centrifugation from yeast-extract glucose medium and were suspended in distilled water. Each series was carried through four serial transfers. There is evidence of adaptation to the ammonium sulfate medium, particularly by cultures 9W, 11W and 23W. These cultures show increased acidity with increasing inocula in each of the four transfers. With a given size of inoculum the acid formed increased with the number of transfers up to the fourth when the acid was about the same as in the third. Increased growth during serial transfer indicated that the bacteria were becoming acclimated. Apparently the ability to synthesize amino acids increased during each transfer. The growth was uniform on serial transfer when amino acids were supplied (table 1). Presumably this adaptation or "training" occurs more readily when a large inoculum is used. It does not seem probable that the increased growth is due directly to the material from the yeast extract which is carried over; if this were true and adaptation did not occur, growth would decrease rather than increase as the material was depleted by serial transfer. It is probable that the "carry over" facilitates growth on the first transfer and this speeds up adaptation by the organism. Cultures 39W, 52W and 53W showed little difference with size of the inoculum or number of transfers. Previously 9W, 11W, 23W, 39W and 53W failed to grow with one drop of inoculum in this medium (table 3). No explanation can be given for the change. It illustrates an alteration in the nutritional requirements of bacteria; in this case the conditions influencing the change were not controlled.

Dispensability of ether extract of yeast extract, vitamin B_1, and riboflavin in ammonium sulfate medium

Different combinations of the three factors were tested with eight cultures in serial transfer (table 6). There was considerable variation in the response of different cultures, which may be due in part to the nutritional state of the organism, i.e., its power to synthesize essential substances at the time studied. Cultures 35W and 49W have been the most consistent in their ability to grow in ammonium sulfate medium, but 52W and 53W grew more vigorously after they were "trained" to this medium. Cultures 9W, 11W, 23W and 39W experienced more difficulty in growth. A different sample of ether extract of yeast extract was used and it was not as effective as the sample used in the experiment of table 5. Briefly, the results in table 6 show that the ether extract of yeast extract is sufficient stimulant by itself to allow growth of 35W, 49W, 52W and 53W. The latter two cultures showed no further stimulation by additional factors. They may be able to synthesize or dispense with these factors. Ether extract of yeast extract was essential for all cultures, as shown by the absence of growth in medium containing only B_1 and flavin. The combination of the extract of yeast and flavin was no more effective than the yeast factor alone. B_1 shows a definite stimulation in the presence of the yeast factor with cultures 23W, 35W, 39W and 49W. All three factors were influential in promoting growth of 9W, 11W, 23W and 39W. This stimulation by flavin supports the previous results shown in table 3 and provides additional evidence that flavin stimulates growth of propionic acid bacteria. Cultures 35W, 49W and 52W apparently acquired the ability to synthesize flavin or dispense with it during growth in ammonium sulfate medium. Conditions controlling the development of this ability are not fully understood, inasmuch as the stimulating effect of flavin could not be uni-

TABLE 5

Effect of size of inoculum on acid production by propionic acid bacteria

Medium contained ether extract of yeast extract, B_1, riboflavin and no amino acids

SIZE OF INOCULUM	NUMBER OF TRANSFERS											
	1	2	3	4	1	2	3	4	1	2	3	4
	Cubic centimeters of 0.1 N acid per 10 cc. of medium											
	9W				11W				23W			
drops												
1	0.3	1.0	1.4	1.3	0.7	0.9	1.0	1.0	0.3	0.3	1.6	1.6
2	0.7	1.4	1.7	1.9	0.8	1.0	1.6	1.5	0.5	1.1	2.7	2.7
5	1.1	1.7	2.7	2.3	1.1	1.4	2.0	2.1	0.7	1.6	3.2	2.8
	39W				52W				53W			
1	1.2	1.2	1.2	1.2	2.8	3.0	3.3	2.8	2.9	2.8	2.9	2.9
2	1.6	1.5	1.7	1.6	2.9	3.2	3.4	3.2	3.2	2.8	2.9	3.3
5	1.6	1.6	1.4	1.3	3.3	3.3	3.5	3.4	3.3	2.9	2.7	3.2

5-day incubation except 7-day with 23W. Inoculum obtained from yeast extract glucose medium.

TABLE 6

Effect of ether extract of yeast extract, B_1 and riboflavin on acid production by propionic acid bacteria

MATERIAL ADDED TO BASAL MEDIUM*	NUMBER OF TRANSFERS																			
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	Cubic centimeters of 0.1 N acid per 10 cc. of medium																			
	35W					49W					52W					53W				
Y	2.4	1.7	2.2	2.4	†	2.3	2.2	1.6	1.9	1.9	4.2	4.5	3.4	4.4		3.4	1.9	3.9	3.5	†
B_1F	0.1					0.1					4.1	0.1				3.9	0.1	0.1		
YF	2.4	1.4	0.3	2.9	†	2.7	2.3	0.9	2.1	0.8	3.8	3.4	3.3	†		3.9	3.5	3.3	†	
YB_1	4.2	2.4	3.5	3.4	†	4.5	4.5	4.7	4.6	4.5	4.4	4.0	3.1	†		3.5	3.4	3.3	†	
YB_1F	4.5	2.2	3.4	3.5	†	4.6	4.6	4.4	5.0	4.7	3.9	3.3	3.1	†		3.9	3.7	3.3	†	
	9W					11W					23W					39W				
Y	0.7	0.2	0.1			0.5	0.1	0.1			1.0	0.3	0.2			0.5	0.1	0.1		
B_1F	0.9	0.1				0.5	0.1				1.0	0.1				0.8	0.1			
YF	0.7	0.3	0.2	0.2		0.5	0.3	0.2	0.2		0.9	0.3	0.2	0.2		0.7	0.1			
YB_1	0.8	0.4	0.3	0.3	0.2	0.5	0.3	0.3	0.2	0.1	1.0	0.9	0.4	0.7	0.5	0.8	0.7	0.5	0.8	1.4
YB_1F	0.9	1.1	1.0	1.4	1.3	0.7	0.9	0.7	1.3	1.3	1.3	1.1	0.9	1.3	1.0	0.9	1.0	1.0	1.1	1.4

7-day incubation except 5-day with 35W and 49W. Inoculum obtained from supplemented synthetic medium.

* B_1 = 0.1 gamma vitamin B_1, F = 6.0 gamma riboflavin, Y = ether extract of 0.3 grams of yeast extract, per 10 cc. medium.

† Not transferred.

formly demonstrated with cultures taken directly from the stock cultures in yeast extract medium.

Another instance of change in nutritional requirements may be cited in which culture 49W was "trained" to grow as well in the absence of vitamin B_1, as in its presence. This adaptation occurred after continuous serial transfer in ammonium sulfate medium containing only the yeast factor as a stimulant. The titrations following the five shown in table 6 were 6th = 1.0, 7th = 2.6, 8th = 2.8, 9th = 3.0, 10th = 3.6, 11th = 4.8, 12th = 4.8. Apparently the culture gradually acquired the ability to dispense with vitamin B_1.

It has been known that the nutritional requirements of bacteria can be changed by alteration of the substrate. The literature has been reviewed by Knight (1936). In the present work we have been able to "train" cultures to dispense with amino acids, flavin and vitamin B_1.

Replacement of yeast factor by other stimulants

Knight (1937) has shown that nicotinic acid and vitamin B_1 are able to replace the *Staphylococcus* factor and in addition Richardson (1936) has shown that uracil is necessary for anaerobic growth of certain staphylococci. Mueller and Cohen (1937) have found in the case of the diphtheria bacillus that beta alanine, nicotinic acid and pimelic acid are able to replace almost completely the growth factor obtained from liver concentrate. Combination of these factors together with pantothentic acid, kindly furnished by Prof. R. J. Williams, were not able to replace the yeast factor when used in hydrolyzed casein medium containing riboflavin and vitamin B_1. Three concentrations were tested as shown below with cultures 35W and 49W. Quantities are expressed in gamma per cubic centimeter of medium.

	1	*2*	*3*
Pantothentic acid	0.02	0.2	2.0
Uracil	0.3	5.0	30.0
Nicotinic acid	0.1	2.0	10.0
Pimelic acid	0.1	2.0	10.0
β alanine	0.1	2.0	10.0

There was some growth on the first transfer but none in subsequent transfers. It is probable that some of these factors are active stimulants for the propionic acid bacteria but the absence of one of the essential factors found in yeast extract prevents growth.

DISCUSSION

Although Tatum, Wood and Peterson (1936b) and Fromageot and Laroux (1936) found that the propionic acid bacteria are able to utilize ammoniacal nitrogen, the present investigation is the first in which these bacteria have been grown during numerous transfers in ammonium sulfate medium with vigorous growth comparable to that obtained in the presence of amino acids. Previously Wood, Tatum and Peterson (1937) had observed inconsistent growth in ammonium sulfate medium during serial transfer. The factor obtained from polenta by Fromageot and Laroux probably is not free of amino acids. The yeast factor is believed to be free of all amino acids. It is obtained by extraction with ether and the material used for this extraction is made strongly acid (pH 2.0). Under these conditions amino acids are not extracted as shown by the ninhydrin reaction. The extract does contain nitrogen, however.

The question of whether or not propionic acid bacteria which are "trained" to dispense with B_1 and lactoflavin acquire the ability to synthesize these compounds cannot be answered at present. It may be that the organisms divert their metabolism so that these compounds are not necessary. As yet we have not tested the medium of "trained" cultures to determine whether vitamin B_1 and riboflavin are formed.

The physiological function of these factors is a subject of interest about which we know little at present. The procedure applied by Lwoff and Lwoff (1937) in the study of the "V" factor with *Haemophilus influenzae* showing it to be a codehydrogenase is instructive and might be useful in a similar study with these organisms. Fromageot and Chaix (1937) have found that compounds of sulfur are important in influencing the critical number of bacteria which are able to activate glucose breakdown. The relation of this factor to those considered here is not clear.

SUMMARY

It has been found that:

1. Amino acids are beneficial but not essential to the propionic acid bacteria. Certain cultures grow with difficulty in their absence, others thrive.
2. Riboflavin (0.05 gamma per cubic centimeter) stimulates growth in ammonium sulfate medium but can be dispensed with by most cultures.
3. Vitamin B_1 is an effective stimulant in ammonium sulfate medium but some cultures can be "trained" to grow vigorously without it.
4. Ether extract of yeast extract is essential for all cultures. It is not replaced by a mixture of nicotinic acid, vitamin B_1, pimelic acid, uracil, beta alanine and pantothentic acid.
5. The nutritional requirements of the propionic acid bacteria are variable, probably depending upon the ability of the culture to synthesize essential substances at the time tested.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

OHIO BRANCH

OHIO STATE UNIVERSITY, COLUMBUS, OHIO, APRIL 30, 1938

STUDIES ON THE MODE OF ACTION OF SULFANILAMIDE. *Robert Finkelstein and Jorgen M. Birkeland*, Department of Bacteriology, Ohio State University, Columbus.

In an effort to explain the mode of action of sulfanilamide and Prontosil in the therapy of hemolytic streptococcus infections, studies were made to determine whether the drugs might act *in vitro* as artificial opsonins. Mixtures of fresh guinea pig leucocytes and hemolytic streptococci were treated with varying amounts of the drugs and incubated for thirty minutes. It was found that in concentrations of from 1:50,000 to 1:100,000 these drugs enhanced phagocytosis. No increased phagocytosis occurred in the absence of fresh serum. When either the leucocytes or the bacteria were treated with the drugs and then washed before mixing with the other no increased phagocytosis was observed. These drugs did not appear to enhance the phagocytosis of *Eberthella typhosa*, *Staphylococcus aureus*, *Brucella abortus* and several types of pneumococci.

PREPARATION OF COLLOIDAL GOLD SOLUTION. *R. D. Scott and Goldie de Long*, Ohio Department of Health Laboratory, Columbus.

In preparing colloidal gold solution, we use the method of Miller and his coworkers (oxalic acid-formaldehyde reduction), except that the reagents are added successively at 50-60°C. instead of at separate temperature stages, and the heating is carried to boiling instead of to 90°C.

The success or failure of preparation is considered largely due to the technic of the formaldehyde reduction which is presented in detail.

No adjustment in reaction is made after preparation; only the behavior with salt solution and with known paretic and normal spinal fluids is noted. Sols of satisfactory appearance thus prepared are, almost without exception, found satisfactory for use in the Lange's test.

DEMONSTRATION OF AN APPARATUS FOR PRESERVING CULTURES IN DEHYDRATED FORM. *George E. Rockwell*, Cincinnati, Ohio.

VITAMIN REQUIREMENTS OF STAPHYLOCOCCUS AUREUS. *Maurice Landy*. Research Division, S.M.A. Corporation, Cleveland, and the Department of Bacteriology, Ohio State University, Columbus.

The experimental work was directed chiefly toward the titration of nicotinic acid and thiamine as growth requirements of *Staphylococcus aureus*, the growth effect of compounds related to nicotinic acid, the testing of 212 strains of *S. aureus* for growth in a completely synthetic medium, and the significance of bios in the nutrition of this microorganism.

Thiamine and nicotinic acid (or amide) are essential in the nutrition of *S. aureus*, no growth occurring in the amino-acid glucose medium alone, or with the addition of thiamine or nicotinic acid separately. Certain compounds related to nicotinic acid and nicotinic amide cannot be utilized by *S. aureus*. To date, 208 typical strains of *S. aureus* have been grown in an amino-acid glucose medium plus nicotinic acid and thiamine.

Vitamin B_6 and vitamin K show no growth stimulating effect when used under the conditions of our test. Bios is not indispensable in the nutrition of *S. aureus*, but the growth stimulating effect of nicotinic acid and thiamine can be considerably increased by its addition.

THE RÔLE OF THE "X" FACTOR AS A REQUIREMENT FOR GROWTH OF HEMOPHILUS INFLUENZAE. *Thomas L. Snyder and R. H. Broh-Kahn.*[1] Department of Bacteriology, College of Medicine, University of Cincinnati, and the Institute for Medical Research, The Jewish Hospital, Cincinnati.

It is generally acknowledged that *Hemophilus influenzae* requires for growth the presence of the so-called "X" and "V" factors. As the result of an extensive investigation into the nature of these factors, Lwoff has demonstrated not only that protohemin is the "X" factor and Coenzyme I or II the "V" factor, but also has concluded that the function of "X" is to supply a respiratory system to the organism which lacks such a mechanism. If the sole function of the "X" factor were to supply a system for oxygen consumption, it should be possible to replace the "X" factor by a reversible oxidation-reduction system of such potential that it can serve as a respiratory catalyst.

Accordingly, attempts were made to grow *H. influenzae* in a medium containing "V" factor with various oxidation-reduction indicators. Cysteine was added to destroy any hydrogen peroxide accumulating as a result of air oxidation of the leuco dye. In the absence of "X", it was found that growth occurred in the presence of the "V" factor plus either a dye and cysteine, or cysteine alone. As a result of this and other findings, it is concluded that the "X" factor functions by supplying catalase to the organism. The theoretical and practical implications are discussed.

RABIES AS A PROBLEM IN PUBLIC HEALTH. *M. C. Hanson,* Department of Health, Toledo.

"CANNIBALISM" AMONG BACTERIA. *Edward A. Steinhaus and Jorgen M. Birkeland,* Department of Bacteriology, Ohio State University, Columbus.

Plate counts of from 5 to 30 million bacteria per cc. were obtained from nutrient broth cultures of a number of common saprophytic bacteria after a year and a half incubation. In an effort to determine whether the bacterial cells themselves were serving as a source of nutriment in such cultures, media were prepared in which the only known source of food was bacterial cells. Solid media were made from heat-killed cells of *Sarcina lutea*, *Serratia marcescens*, *Staphylococcus aureus*, as well as the whole and the disrupted cells of *Escherichia coli*. These media were inoculated with 21 different bacteria. In general, the proteolytic organisms grew best on the various media. Definite zones of digestion were evident. Of the 21 bacteria tested, best growth was obtained with *S. lutea*, *S. marcescens*, *Bacillus subtilis*, *Bacillus mycoides*, *Bacillus anthracis*. Demonstrable but not normal growth was obtained with *E. coli* inoculated on the medium containing *E. coli* cells. The one strain of *S. aureus* used did not support good growth of any of the bacteria tested. *S. marcescens* produced no pigment when grown on cells of *E. coli*, although it did when grown on the media containing other bacterial cells. *S. marcescens* and *S. lutea*, when inoculated into sterile suspensions of the homologous species, gave counts almost as high as in nutrient broth, thus supporting the results obtained on plates of the cell media.

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[1] Eli Lilly and Company Research Fellow.

ANAPHYLACTIC SHOCK PREVENTED BY KETENIZING SERUM. *J. T. Tamura and M. J. Boyd*, College of Medicine, University of Cincinnati, Cincinnati.

Ketene was produced by the thermal decomposition of acetone vapors. In general, the reactions of ketene are addition reactions with the amino groups of proteins at neutrality, which enables one to acetylate proteins, properly buffered, without denaturation.

Buffered anti-Brucella horse serum was treated with ketene for various lengths of time. Guinea pigs sensitized to the original anti-serum received intracardially a shocking dose of ketenized anti-serum. Sensitized control animals received the original anti-serum by the same route. The control animals went into shock and died, but guinea pigs receiving anti-serum ketenized for 35 minutes or more showed no anaphylactic syndrome or very mild signs of shock. The ketenized anti-serum retained the major part of its agglutinin antibody.

THE TREND OF SYPHILIS IN CLEVELAND. *James A. Doull and Huldah Bancroft*, Department of Hygiene and Bacteriology, Western Reserve University, Cleveland.

Between 1921 and 1935, 27,976 women were given Wassermann tests in the Prenatal Clinics of Maternity Hospital of Cleveland. The six to eight clinics served nearly the same area from year to year and were fairly wide-spread over the city. First admissions were analyzed separately from re-admissions, as persons found positive on first admission were referred for treatment. For first admissions, percentages positive for white women range from 2.2 to 4.1; for colored women 7.6 to 15.6. The total admissions show in every case a somewhat lower percentage of positives than do re-admissions. For white women there is evidence of a downward trend since 1925. For colored women there is a definite upward trend.

MULTIPLICATION OF VACCINIA VIRUS IN DEEP COLUMNS OF MAITLAND MEDIUM, *Margaret S. Coates and R. L. Thompson*, Department of Hygiene and Bacteriology, Western Reserve University, Cleveland.

By carefully controlling the tissue-fluid ratio, growth of vaccinia virus was obtained in columns of Maitland medium with a depth of over 45 mm. The New York City Board of Health strain of virus was carried through 25 serial transfers in deep cultures without loss in virulence.

PROPAGATION OF THE HUMAN INFLUENZA VIRUS IN THE GUINEA PIG

Fetus.[1] *Oram C. Woolpert, Fred W. Gallagher, Leona Davis, and N. Paul Hudson,* Department of Bacteriology, Ohio State University, Columbus.

The P.R. 8 strain of human influenza virus was inoculated intracerebrally into fetal guinea pigs *in utero*. It was found to multiply and disseminate widely in fetal tissues, attaining high titers particularly in lungs, liver, and placenta. Forty-eight hours proved to be a favorable incubation period. By subinoculating with lung tissue, two series of passages through fetal guinea pigs have been accomplished, one of 10 transfers, the other of 16; the latter is still in progress. The identity and virulence of the virus were maintained during passage, as shown by appropriate tests in mice. It was found that when the virus titer of passage material had been greatly reduced by storage, or otherwise, it could be quickly restored by further passage. It is concluded that the fetal guinea pig is a favorable animal for experimentation with this virus and that large quantities of bacteriologically sterile virus can be made available by the technic described.

Adsorption of Poliomyelitis Virus by Cholesterol, Ergosterol, and Other Sterol Products. *J. A. Toomey and W. S. Takacs,* Department of Pediatrics, Western Reserve University, Cleveland.

These workers previously demonstrated that cholesterol adsorbs poliomyelitis virus. They then took related products, ergosterol and primary crystalline vitamin D, and were able to demonstrate that these also either adsorbed or inactivated poliomyelitis virus in the same manner as did cholesterol. Their method was to add 10 per cent ergosterol or primary vitamin D to a 1 per cent suspension of virulent virus, grind the combination in a ball mill, and inject the supernatant fluid left after centrifugation into *Macacus rhesus* monkeys. They ground the same amount of virus in physiological saline and injected the suspension into control animals.

Pathology of the Human Influenza Virus in the Guinea Pig Fetus. (By Title) *N. Paul Hudson, Fred W. Gallagher, and F. S. Markham,* Department of Bacteriology, Ohio State University, Columbus.

EASTERN NEW YORK BRANCH

Rensselaer Polytechnic Institute, Troy, N. Y., April 29, 1938

The Virulence for Mice of Certain Pneumococcus Strains Following Induced Specific Type Transformation. *Albert H. Harris,* Division of Laboratories and Research, New York State Department of Health, Albany.

The relationship of virulence to type specificity was studied by inducing reciprocal type transformation between a type of pneumococcus of high virulence for mice (type II) and one of low virulence for mice (type XIV). The technic was that described by Dawson and Warbasse. A strain representative of the transformation from type II to type XIV was tested for virulence, which was found to be as low as that of the type-XIV strain used in the preparation of the vaccine.

With some difficulty, a type-XIV strain was found which could be transformed to a type II. Virulence of this transformed strain was high, similar to the type-II strain from which the heat-killed vaccine was prepared.

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[1] Aided by a grant from Eli Lilly and Company.

These results strongly suggest that virulence is intimately associated with type specificity *per se*, at least as far as the strains of type II and type XIV which were employed are concerned, since, when the strains underwent specific type transformation, they assumed the virulence of the representative strain which was used in inducing the change to the new type.

THE ACTION OF SOIL MICROÖRGANISMS ON THE CARBOHYDRATES OF PNEUMOCOCCI OF TYPES I-XXXII. *Grace M. Sickles and Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

As an approach to the study of relationships between the various polysaccharides, comparative tests were made of the activity of *Bacillus palustris*, *Saccharobacterium ovale*, and *Saccharobacterium acuminatum* on the carbohydrates of pneumococci of types I to XXXII.

The polysaccharides of pneumococcal types, apparently related immunologically, were not always decomposed by the same strains of soil microörganism. The strain of *B. palustris* which produces an enzyme active against type-III carbohydrate decomposed type-III carbohydrate only. The very closely related strain of *B. palustris* which produces an enzyme that splits type-VIII carbohydrate decomposed that carbohydrate only. *S. ovale*, which was isolated on type-II carbohydrate medium, decomposed the polysaccharides of ten other types but not of type V. Two strains of *S. acuminatum* utilized the carbohydrates of twenty pneumococcal types. The polysaccharides of six types were decomposed by none of our strains.

The following cross reactions in precipitation tests disappeared simultaneously with the loss of precipitative reactivity in the homologous serum: type-II carbohydrate in types V and XX sera; type III in type-VIII serum; type VIII in types III and XIX sera; type XVI in type-XXVIII serum; type XXVIII in type-XVI serum; type XX in types X and XXXI sera, and type XXXI in type-XX serum.

PRELIMINARY REPORT ON THE ACTION OF IMMUNE SERUM IN CONJUNCTION WITH SULFANILAMIDE IN TYPE-III PNEUMOCOCCUS INFECTIONS IN MICE. *John K. Miller*, Division of Laboratories and Research, New York State Department of Health, Albany.

The effect of specific antibacterial serum, of sulfanilamide, and of serum combined with sulfanilamide therapy on pneumococcemia in mice was studied with 10 to 10,000 M.L.D. of a recently isolated and a stock mouse-passage strain of type-III pneumococcus. Sulfanilamide totaling 61.6 mg. was given to each animal, subcutaneously, over a period of five days: 17.6 mg. a day for two days and 8.8 mg. a day for three more days. Specific antibacterial rabbit serum alone in adequate doses afforded complete protection. With sulfanilamide alone, all animals were dead within ninety-six hours. Combined therapy with the above schedule gave variable results and in some experiments was not so effective as the use of serum alone.

However, when the schedule of dosage was varied so that 60 mg. of sulfanilamide were administered in two days, combined with serum which alone permitted 10-per cent survival, all animals survived approximately ninety-six hours, and 30- to 60-per-cent protection against the stock strain and 30 per cent against the more recently isolated strain was obtained. With sulfanilamide alone, there was 100-per-cent mortality in ninety-six hours, but life was prolonged forty-eight hours longer than in the controls.

A STUDY OF MENINGOCOCCAL CULTURES FROM HORSES IMMUNIZED AGAINST MENINGOCOCCI. *Sophia M. Cohen*, Division of Laboratories and Research, New York State Department of Health, Albany.

Meningococci were isolated from eleven horses which developed endocarditis during immunization against several group I-III and group-II meningococcal strains which varied widely in virulence and other biologic characters. Forty-seven cultures were recovered from the blood or from lesions on the heart valves.

All cultures from ten horses were identified as group I-III; that from the eleventh, isolated only at autopsy, was mixed but group-II microörganisms predominated. No changes were observed in the agglutinative or precipitative activities of the meningococci isolated repeatedly from individual horses during the course of the infection. Those from eight of the horses showed a striking similarity to one particular stock group I-III strain used in immunization, which suggested that this strain is exceptionally well-adapted to survive and grow in the tissues.

The cultures studied from four of the horses exhibited a low degree of virulence for mice; those from the fifth a high degree, but after treatment of this animal with sulfanilamide a decrease in virulence was noted. Capsular swelling of the cultures tested was slight or absent as in the case of certain of the stock strains; viability in sodium-chloride solutions, in general, was marked. Cultures with these properties may possibly represent a phase in the dissociation of the meningococcus.

PURIFICATION AND ANALYSIS OF DIPHTHERIC TOXIN BY ULTRAFILTRATION. *Mary W. Wheeler*, Division of Laboratories and Research, New York State Department of Health, Albany.

With the strain of diphtheria bacillus used in these studies—No. 3203—toxins with a flocculation value of from 10 to 14 and an M.L.D. of from 0.003 to 0.004 cc. were regularly produced in a synthetic medium in which the only sources of nitrogen added were aspartic acid and cysteine hydrochloride.

By ultrafiltration of culture filtrates through 8.5- or 9.5-per-cent Parlodion membranes, it was possible to obtain purified, concentrated residues which contained practically all of the proteins and toxin synthesized by the original culture. The nitrogen content varied from 0.001 to 0.002 mg. per Lf.

These residues could be separated by refiltration into two fractions: one, the portion retained by a 4.5-per-cent Parlodion membrane; the second, the residue obtained by reconcentrating on a 9.5-per-cent membrane the filtrate and washings from the 4.5-per-cent membrane.

The first fraction was relatively nontoxic and contained the proteins precipitable with 33-per-cent saturation of ammonium sulfate. The second fraction contained from 50 to 70 per cent of the toxin. The proteins present were precipitated with from 50- to 60-per-cent saturation with ammonium sulfate, but no precipitate was obtained with 33-per-cent saturation. The nitrogen content approximated 0.0005 mg. per Lf.

FURTHER OBSERVATIONS ON THE TOXIGENIC PROPERTIES OF HEMOLYTIC STREPTOCOCCI. *Julia M. Coffey*, Division of Laboratories and Re-

search, New York State Department of Health, Albany.

Five hundred and ninety-seven cultures of hemolytic streptococci were classified according to precipitation groups and examined for toxigenic activity; with 450 the virulence for mice was determined. The highest incidence of virulent strains occurred in group C-G. Although toxigenic activity as demonstrated by the cutaneous reactions induced in rabbits was largely limited to group A, a small number of group C-G cultures from horses produced toxins.

The toxigenic strains were divided into groups on the basis of toxin neutralization by univalent horse sera of three selected group-A strains. These sera alone or in combination neutralized the toxins of all of the strains of groups A and C-G. A multivalent horse serum produced with two strains of different toxin groups neutralized the toxins of 147 group-A strains which included representatives of the various toxin groups.

This study reemphasizes the complex character of the antigenic activity of streptococcus toxins. Within one toxin group marked differences were demonstrated by neutralization tests with several heterologous antitoxins and by studies of antigenic activity in goats and rabbits. The number of toxin groups defined is thus dependent upon the valency of the antitoxins used in classification. Furthermore, the antigenic valency of a toxin is in some instances modified by the animal species in which the antitoxin is produced.

ALICE IN VIRUSLAND[1]

PAUL F. CLARK

Medical School, University of Wisconsin, Madison

(My apologies to Lewis Carroll with the hope that he will extend his usual gracious blessing to this latest parody on his immortal "Alice.")

PROLOGUE

Since this particular President has had only eight months in which to ripen instead of the usual twelve, you must expect his remarks to be a little green, possibly even somewhat tart. If they should prove too acid, I beg you to expose them to the warm California sun to supply sugar and a richer flavor. As a matter of fact, I wrote another address, "Immunity in Poliomyelitis," for this occasion, but it was so heavy that the thought of a long day of meetings in the summer followed by a sumptuous dinner decided me to discard the heavier in favor of a lighter medium; so I pray you, please bear with me and be generous. May I add for the benefit of the ignorant ones that my wife's name is also Alice and that she works at times with me in the laboratory. So if on occasion "our Alice" should make a grown-up remark you will realize that she has a multiple personality.

Alice was getting tired of waiting for her father; she was sitting in the one comfortable chair in the laboratory wondering *why grown-ups always had to finish things*, when suddenly a small monkey jumped out of the cage, dashed by her, and popped down the ventilating shaft. In a flash, down went Alice, trying her best to catch the runaway. Down, down, she went; it seemed like miles. The monkey was always just ahead of her and she could read his name "Ferdinand" on his collar. "I wonder," she thought, "if Ferdinand isn't going back to India to find his mother. Wouldn't that be fun! I should love to see that river they talk so much about, where the people all go to say their prayers. Let me see; is it the Styx or the Jordan? Oh no, I remember, it's the Ganges."

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[1] Presidential address delivered before the Society of American Bacteriologists at its fortieth annual meeting, San Francisco, August 31, 1938.

A blurred jumble of thoughts and hours passed as Alice hurtled on through the earth, when suddenly, ke-splash,—down she plopped into deep water. *Such* a douche! And *such* a plunge! But she swam hard, managing to come to the surface at last and to get to the shore just in time to see Ferdinand dash across the beach to the woods. After him raced Alice, only to see him jump into the trees, saying as he went, "Damn his tail and whiskers, I know I shall be late to the tea."

"That reminds me," thought Alice. "It *is* getting late and I *am so hungry*." As if they could read her thoughts, the monkeys threw down a shower of bananas from above, and Alice scrambled around picking up several. "Mmmmm, this is delicious!" said Alice, looking about at the foreign scene as she munched the ripe fruit.

"But what a curious feeling! I must be shutting up like a telescope!" She had eaten so heartily that the effect was immediate; by this time she was only three inches high. "I have heard of a bird's-eye-view," said Alice to herself somewhat frightened, "and I surely am getting one now. I do hope there is no cat about. He might be tempted."

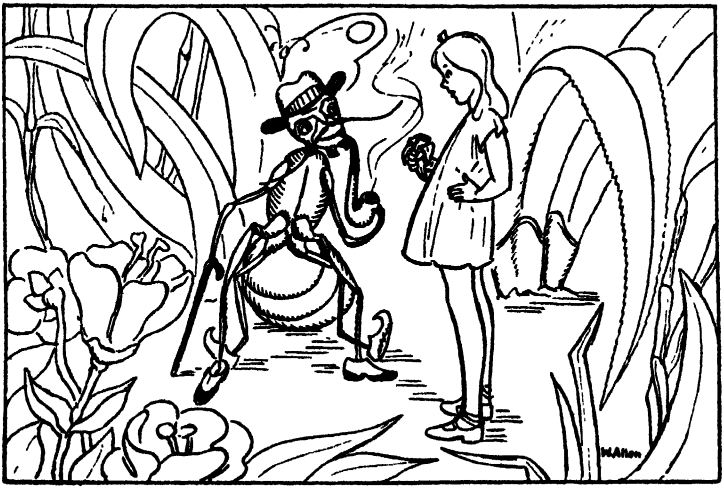
She continued to shrink until she found herself looking in a neighborly fashion into the curious eyes of a large black ant who was comfortably puffing away at a briar pipe. "Curiouser and curiouser," thought Alice. "I suppose I am now getting an insect's view of things. I hope no bird will think me suitable for his afternoon tea," remembering with distress the ants she had sometimes trod upon. "I certainly shall watch my step when I get home again."

"How different one does feel when one is small and weak!" said Alice aloud.

"What do you mean,—small and weak?" asked the Ant, in a gruff voice. "Why, the strongest and most sensible creatures I know are the smallest; just think of bacteria and the filterable viruses!"

Now of course, Alice, being her father's daughter, knew that bacteria and viruses cause many diseases, but she was astonished to hear the Ant discuss them as though they were rational beings. At a loss, she responded with the only polite remark she could think of, " 'Go to the ant, thou sluggard; consider his ways and be wise.' "

Whereupon the Ant, with a grin of pleased satisfaction on his bulbous face, removed his pipe and murmured, " 'Out of the mouths of babes and sucklings!' Who are you, anyway?"



Alice in Conversation with the Black Ant

Alice replied rather shyly, "I—I hardly know, Sir, just at present—at least I know who I was when I got up this morning, but I have changed so much since then—"

"Oh, so you're a changeling!"

Alice did not like this suggestion, so she turned away, absentmindedly nibbling more of the banana which she still held in her hand. Rapidly she dwindled in size until she was smaller than the smallest grain of sand, and the Ant, her erstwhile neighbor, looked like a great irregular hill.

Viewing a world entirely new to her eyes, she was amazed by the minute, crawling, wriggling and tumbling creatures everywhere. The commotion around her, and the bumping of all the colloidal particles together caused loud crashing noises, so that she was terribly frightened and almost stunned by the roar. She managed, however, to find a small sand grain with jagged margins, so that with much effort, she pulled herself up out of the little damp spot where all these creatures were racing to and fro. She now began to look about her with more precision, and discovered out on the drier sand, away from the commotion at her feet, two strange creatures doing a peculiar dance. "What *can* they be?" she said aloud.

To her surprise, a rod-shaped creature with a large head, standing on the back part of *her* grain of sand answered, "What can be your name, and where do you come from, that you do not know two of the most powerful clans of our race?"

"Listen," said the Tetanus Bacillus, without waiting for Alice to answer, "and you shall hear what is troubling them."

THE COCCUS AND THE SPIROCHAETE

The Coccus and the Spirochaete
Were walking down the strand.
They choked upon a starfish;
"We simply cannot stand
Such large and clumsy proteins
Who think they are so grand."

"If trypsin strong could aid us now
By splitting him in twain,
Do you suppose," the Coccus said,
"T'would give us awful pain?"
"I doubt it," said the Spirochaete,
"There's too much tryptophane."

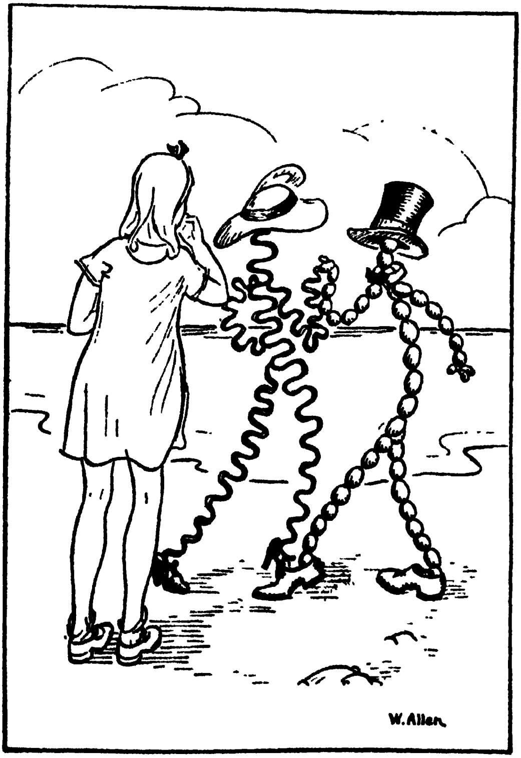
"The time has come," the Coccus said,
"To talk of many things,
Of stools and stains and stigmata
Of guinea pigs and goats.
And why the neutron has no charge
And whether iron floats."

Forward came the anaerobes
Moving in stately dance,
While spirochaetes on every hand
Wriggled a lively prance.
And all the little "viri" stood
Arrayed in purple pants.

"But wait a bit," the Coccus cried,
Deciphering his notes,
"This human race is surely mad
They slit each other's throats.
They blow each other off the earth.
They sink each other's boats."

"Should we permit," the Coccus asked,
"These silly beasts to thrive,
Who call themselves the lords of earth
Yet selfishly connive
Freedom, dignity and peace
From many lands to drive?"

Spake Influenza Virus then,
"Let's smite them hip and thigh,
They're clever, strong, and hard to beat,
But smack them in the eye.
If we combine in our attack,
Surely they must die."



THE COCCUS AND THE SPIROCHAETE ESSAYED A STATELY DANCE

"*Whatever do they mean?*" said Alice.

"Oh, they have called the Microbe Parliament together to consider what to do with 'man.' Come to the meeting with me this evening and you shall hear. Representatives from all the clans will be there."

"Will the king and queen be there?" asked Alice timidly.

"We deal not with dictators or kings," said Clostridium. "We be free people; free to gather, free to come and go, free to live our own lives without decrees from others."

As dusk came on, Alice and the Clostridium walked along, hand in hand, until they came with countless other strange beings to a great cave in the rock, with the roof seemingly supported by rows of stalagmites joining the stalactites from above. As they entered, Alice noticed that masses of small rods were gathered around each column and that each of these rods glowed from within with a pale greenish light, so that the whole cavern was beautifully illumined, with the rays reflected from every crystal.

"How very beautiful!" said Alice. "But these strange creatures! It is quite unbelievable!—Why, some of them look like parallelopipedons with peculiar arms and legs sticking out at all the corners! Are they all alive?"

"Alive?" said the tetanus bacillus. "Oh, we smallest folk never make that inaccurate distinction between life and death which you Gargantuans consider so important. Everything is alive. To be sure, there are different degrees of reactivity and of the excited states."

"Now, over there on the extreme left are some of the markedly excited clans. You call them radio-active substances. A little nearer us are the vitamins; only, of course, we don't give them your peculiar alphabetical names. Let me see, you have about fifty-seven different varieties, haven't you? There is Nicotinic Acid. See him, standing up on his nitrogen, waving his carboxyl tentacle. In front of the vitamins are,—what do you call them? —oh yes,—the hormones. Potent creatures! That clumsy fellow is Testosterone and just beside him is one of the chief causes of cancer. When your pathologists stop quarreling about the origin of this and that malignant cell, and dig harder at the physiology of the cell, they'll get somewhere. But don't let them get so excited over what is animate and what is inanimate.[2] Take my word for it, that notion will go the way of the Ptolemaic Theory of the Universe."

"Oh," said Alice, in a very subdued voice.

"The only distinction *we* attempt to make is to keep the smaller, more active ones over here on the left where they won't disturb the more conservative clans, like my own over here on the right. See that trouble-maker sneaking over to the right; that's a haptene looking for a protein to give it a reputation. And do you see that most peculiar fellow way over at the extreme left, beyond the radio-active substances?"

"Yes", said Alice, "Why, it looks like a constantly changing interrogation point with a lot of funny arms sticking out everywhere. What is it?—or, --or, should I say, who is it?"

"Well," said Tetani, "that is man's best friend or his worst enemy, depending on your point of view. That is the virus of 'Satiable Curtiosity';—he doesn't stay around here very much, he really prefers the company of you Gargantuans."

Alice was almost overcome by the overwhelming scene, but Clostridium said they must hurry to their places as the meeting was about to begin. Straight up the central aisle to a rocky platform at one end, they marched, the Tetanus Bacillus leading the reluctant Alice by the hand. He seated her on a rose quartz crystal on the right of the stage.

In the center, seated on a large sapphire, was a peculiar-looking horseshoe-shaped animal which Alice recognized from her seashore experiences as that curious relic of past ages, the king crab. Her guide explained to Alice that Limulus was retained as chairman and judge of their proceedings because of his long line of illustrious ancestors and his *real blue blood.* "His family is almost as old as mine," said Tetani proudly.

Alice was speechless at the weird scene. Out on the floor of the cave was a great log jam of the strangest creatures. Opposite her in an enclosure on the platform were twelve solemn-looking microbes, good and true, that appeared to be samples of the mass out on the floor.

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[2] This idea is well presented by N. W. Pirie in an essay published in Perspectives in Biochemistry (1938) edited by Needham and Green.

"Most peculiar faces," muttered Alice. "That strangest moon-faced one with long whiskers must be the *Jabberwock.*"

"Not at all," said Tetani, deeply offended. "That is Giardia lamblia, one of the wisest members of the jury."

"Silence in the court!" shouted the Anthrax Bacillus, standing up beside Limulus.

"Read the accusations," said Judge Limulus. Whereupon, the Streptococcus unrolled a parchment scroll on which were inscribed the itemized accusations against mankind:

"Through the skill of a clever member of the Bandar-log, we have with us this evening, a representative of the human race!" Pointing to Alice, he shouted, "She must answer to the charges!"

Alice buried her face in her hands, while the Coccus proceeded to list the accusations, most of which fell on her dazed mind as on deaf ears. She did, however, arouse herself sufficiently to hear the last few words as he summed up his appalling denunciation:

> "Brutality and blind-spots.
> Power to the panderers and the paranoiacs.
> Blind selfishness and blundering sentimentality!"

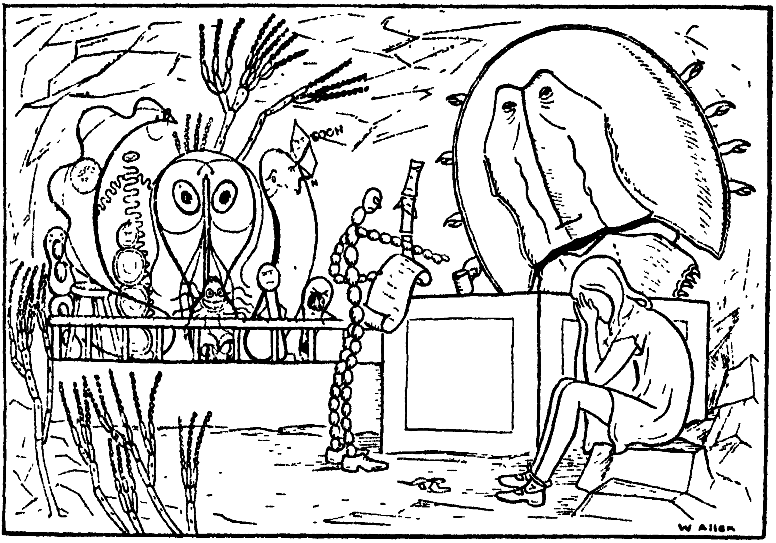
"Would you believe it," said the Coccus, "they build great structures for the care and preservation of the feeble-minded and permit these poorest strains to reproduce their kind!"

Alice continued to sit with buried face while the judge called for the evidence.

"For my part," said the Influenza Virus, "man seems too weak to worry about. With the help of members of some of our other clans, I destroyed millions of them a few years ago and I can do it again any time. War and resulting famine are our greatest aids, and their demagogic leaders easily inflame them to war. We have nothing to worry about. Man is foredoomed to destruction by his own folly."

"I am not so sure," said Corynebacterium diphtheriae. "My family used to enjoy excellent accommodations in the throats of man, but recently, because of some vaccination procedure they have developed, we find it almost impossible to gain a throat-hold. Then, too, they heat their milk to such a temperature that our progeny are destroyed. It is a *'recession'* at least for us, although we *may* be able to meet it temporarily by 'inflation' while developing more virulent strains."

Eberthella typhi urged immediate action. "They heat their milk, they put some killing stuff in their water, and they have developed some way of destroying us inside their bodies. Why, in some cities, our immediate family no longer appears on their ledgers."



THE HUMAN RACE, IN THE PERSON OF ALICE, BEFORE THE TRIBUNAL OF THE MICROBE PARLIAMENT

"Off with her head! Off with her head!" came the cry.

"And the bacteriophage will git you if you don't watch out" shouted somebody in the audience.

Spoke up the Streptococcus again, "I had intended to keep my people out of this discussion, because we have always been so powerful, but I am here to say that recently, man has devised a cunning minute torpedo which 'gets' many of us. We do not feel so completely sure of ourselves as in former years. Maybe man is more capable than we used to think."

Treponema pallidum joined in. "Of course, you understand that for obvious reasons we have always had the inside track, but man has been sending stronger and stronger torpedoes after my relatives in recent years. If human beings really put their minds to it, I believe they could come *pretty close* to wiping out many of our *more highly-specialized* microbe families."

A chant arose from a portion of the audience:

"Wriggle, wriggle, Spirochaete,
How they wonder what you eat,
Down the vessels as you go
Bringing humans pain and woe.
Perhaps you favor glucose sweet
Or carotene you'd rather meet?
But watch your step, when histamine
Gives place to strong arsphenamine."

The next witness was the Tubercle Bacillus who said that his people, too, were hard put to it, and were unable to maintain themselves in the luxury their ancestors were accustomed to. "I second the motion of the Streptococcus that we all combine to elminate mankind."

Cheers from the audience and more cries of "Off with her head!"

Poliomyelitis virus spoke up, "That's all very well for you common laborers with no specialized training. But what would happen to my family if man should be eliminated? We should be wiped out, too. We live through man alone. It strikes me that you began this meeting on a very lofty plane—man should be eliminated for the welfare of the universe, because he is so stupid and immoral,—but what you really want is to save your own jobs and your own skins, just like all the politicians. As a matter of fact, Mycobacterium tuberculosis hominis and several others that have spoken would be in a bad way if man were eliminated. You are all just as selfish and short-sighted as is man. If you would but learn to adapt yourselves to man's physiology, as *we* have almost succeeded in doing, and not destroy him, *he* would not develop means of eliminating you. He is not such a bad fellow if you know him as I do. I hold no brief for members of the human race. Of course, they are weak and selfish and gullible; like us, they are controlled by their emotions instead of their brains. Occasionally even some of their scientists drift over into mysticism and the metaphysical, and such a mess they make of it."

Chorus from the jury box:

"Oh let us never, never doubt
What nobody is sure about!"[3]

"Off with her head!" shouted some.

"Let her explain! Let her explain!" yelled others.

"She can't explain anything," bellowed the Judge, pounding the table for order. "I suggest that you consult the shades of some of the humans that have had sense enough to study *us*. They must surely be the wisest. Suppose two or three of you take Alice over to the Styx and see what some of those old fellows think of their species by now and what they think of your plan. There is one old codger[4] who sits all day in the sun squinting through a little hand magnifying glass and another long, skinny, bearded fellow, a recent arrival,[5] who looks through a big binocular microscope. See what they have to say."

Such an uproar broke out at this suggestion! Each microbe in the assembly tried to address the chair at once. "Just like the legislature!" thought Alice.

During the commotion, two of the least excited jurors came over, took Alice by the hand, and shuffled hastily out the back way, followed by the faithful Clostridium tetani. Outside, all four of them climbed on the back of an enormous fruit fly (Drosophila) who, at the word, sailed off across country with them.

Alice heaved a sigh of relief to be free from the commotion of the trial, even though she knew nothing of her present destination save what the Judge had suggested. Never had she been so insulted and dragged about.

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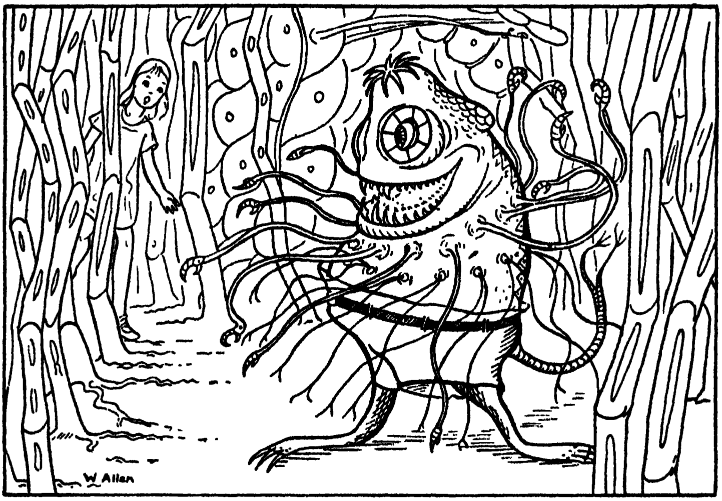
[3] H. Belloc, 1925, J. Path. & Bact. 28, IV.

[4] Antony van Leeuwenhoek.

[5] Theobald Smith.

As they "whiffled" madly on "through the tulgey wood," she began to get her breath and to look about her.

Her two new neighbors were not at all reassuring. One looked much like her chief accuser, the Streptococcus, save that he had on a thick fluffy overcoat of the smoothest material; he sat up very straight and severe as if he were much impressed with his own importance. "He is a Type III Pneumococcus and very powerful," whispered Alice's mentor, Tetani. The other creature, Alice recognized as the Influenza Virus that had spoken so slightingly of the human race. He was a forbidding monster.

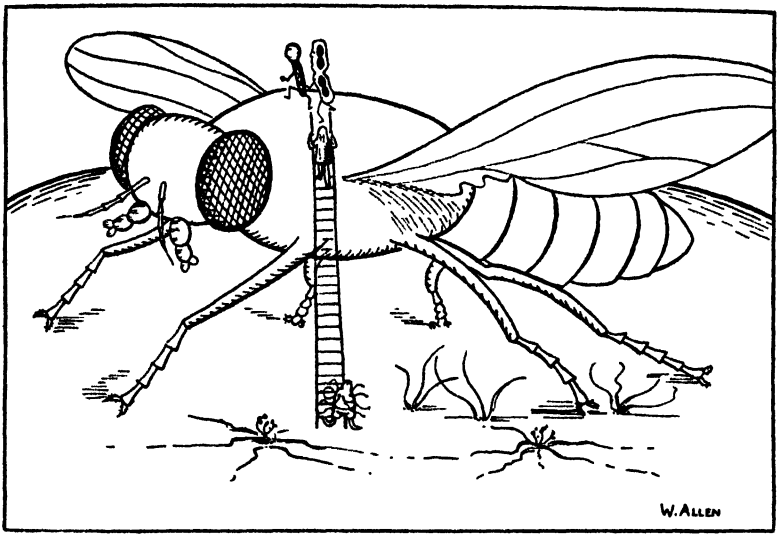


Alice's First Meeting with a "Virus"

Four rows of talons lay beneath
Full twenty pair of canine teeth,
His fork'ed tail stuck out behind
With horny scales of every kind.
His eyes were red and white and green,
Changing with the shifting scene.
His nether limbs? You look askance!
Were covered still with purple pants.

"He is so ugly that he is really quite attractive," said Alice to herself, "but I should hate to get near him. With all those tentacles, he could seize dozens of me at once."

The Fly now landed them gently near a great river with many boats moored along the bank, and others going back and forth from shore to shore. "This must be the Styx," thought Alice. "Yes, it is, because on the deck of that nearest boat are the two old men the Judge was describing."



AIR TRANSIT: VIRUSLAND TO THE STYX

"Come," said Tetani, and together they climbed down a convenient spider web on to a pile of milkweed down. The Pneumococcus told Alice that she must interview a number of these scientists as the Judge had suggested. "You will drink some of the milk from this plant and that will make you grow to your normal size. The Tetanus Bacillus and I will be in your right and left ears respectively to tell you what to say and the Influenza Virus will be in your nose to bite you if you do not say the right thing."

Alice drank deeply of the milkweed juice and immediately began to grow in every direction. The Fly no longer seemed such a huge creature, and soon she was looking *down* on ants and violets as on other days, before she chased the monkey down the ventilating shaft. Her vision, however, seemed much more acute than formerly.

"Ouch!" said Alice, as she felt a sharp nip in her nose, and heard a voice in her left ear bidding her go aboard the houseboat.

There she found the older man with a silvery wig, excitedly looking at something the tall, lean, bewhiskered fellow was demonstrating through his big binocular microscope.

"Marvelous! Wonderful! You *are* right! Just like the picture in this book!"

"I must say to you, as I've oft-times said already, that 'tis not my intention to stick stubbornly to my opinions, but as soon as people urge against them any reasonable objections, whereof I can form a just idea, I'll give mine up, and go over to the other side; and especially because my efforts are ever striving towards no other end than, as far as in me lieth, to set the Truth before my eyes, to embrace it, and to lay out to good account the small Talent I've received; in order to draw the World away from its Old-Heathenish superstition, to go over to the Truth, and to cleave unto it."[6]

"Yes, I see every coil, and you call that a spirochaete? But what a beautiful instrument of precision your microscope is! My little hand lens which I thought so fine is but a toy compared with this. But I did use my one talent. Tell me, has man improved himself as much as he has his optical instruments?"

"I fear not," said the slender one.

"The human race is in a rather delicate, unstable relation to its environment. Its course is frequently left to chance, in the hands of incompetents, instead of being guided by the coöperation of those best equipped. Only now and then is the voice of reason heard in the rare intervals when the human din subsides long enough for the race to catch its breath. The value of human life is profoundly distorted, and the untiring labor devoted to attempts to save a single life is daily and hourly flouted on the highways. Important devices to protect community health, such as vaccination against small pox, are set aside because the accidental individual death rate resulting from their application may be in the second or third decimal place of a per cent."[7]

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[6] Letter to Rev. George Garden cited by Dobell—Antony van Leeuwenhoek and his "Little Animals," p. 74.

"Hmmmmm" whispered the Influenza Virus to the Pneumococcus. "He isn't overly enthusiastic about his human race, is he?"

"No," said the Coccus, "but they do make marvelous instruments and they write down their thoughts in books for all, who wish, to enjoy, even centuries later."

The Virus tweaked Alice's nose and suggested that she see who might be at the other end of the boat. Near the bow, Alice found a hawk-nosed, keen-eyed genial German Physiologist, Carl Ludwig, and a kindly, well-bearded old gentleman, a brother physiologist from Russia, Pavlov, poring over the description and the results obtained with the latest ultracentrifuge. "Grossartig —kolossal—ein Baustein!" cried Ludwig. "Die Methode ist alles! Die Methode ist alles!"[8]

"Yes, yes!" said Pavlov, "but consider the centuries in which this instrument was being developed." Turning to Alice, he said, "I beg you, bear this message to the scientists of your country—'Gradualness, gradualness, and gradualness. From the beginning of your work, school yourselves to severe gradualness in the accumulation of knowledge. Learn the ABC of science before you try to ascend to its summit. Never begin the subsequent without mastering the preceding. Never attempt to screen an insufficiency of knowledge even by the most audacious surmise and hypothesis. Howsoever this soap-bubble will rejoice your eyes by its play, it will inevitably burst and you will have nothing except shame."[9]

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[7] Parasitism by Theobald Smith, Princeton Press, 1934, p. 170.

[8] "Die Methode ist alles" was a favorite thesis of Ludwig.

[9] A part of Pavlov's Testament to the youth of his country written just before his death, 1936.

Much impressed, the Coccus remarked to the Influenza Virus, "These Gargantuans with their written language, the high ideals of their philosophers and scientists, and their printed books, must surely be more effective than we can ever hope to be. They should be able to inspire and teach everybody."

"Humph!" said Influenza Virus. "Did not the 'lean one' say that their leaders were frequently incompetent? We can divide and multiply, can't we?"

"Yes, but we have no enterprise, no memories of the past, no record of the achievements of great individuals of power and spirit to stimulate the whole race."

"We can work the more effectively," grunted the Virus.

Said the Coccus, "We can accomplish great tasks, but we are not individuals; we are merely masses. We do not even know our own parents."

"Pooh," said the Virus. "Do you see those two men over on the next boat? Well, that fellow with the van Dyke beard is the greatest poet and seer of them all; he states of mankind that 'It is a wise father that knows his own child.' And a keen woman essayist from Philadelphia, Agnes Repplier, puts it even more sharply when she says that 'maternity is a matter of fact, while paternity is a matter of conjecture.' Even Landsteiner's contributions and skill merely narrow the limits of the conjecture."

Alice interrupted the dispute by saying, "I recognize Shakespeare, but who is the other man with the large nose, the shining eyes, and the thin face? He is almost surrounded with books. Did he write all of them?"

"Yes," said the Coccus, "that is Erasmus, the apostle of the 'book,' of fair-mindedness, of conciliation, and the master satirist and enemy of war and of fanaticism. He lost the struggle in his own period and he certainly would lose it today. But he knew that 'Fanaticism is fated to overreach its own powers. Reason is eternal and patient, and can afford to bide its time.'[10] But the leaven of reason must permeate the whole people in order to be effective."

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[10] Erasmus of Rotterdam by Stephan Zweig, Viking Press, 1934, p. 21.

At that moment, a great bell sounded. Pavlov put down his book saying it was tea-time, and would Alice be so kind as to be their guest; Alice accepted with alacrity. Leisurely they sauntered along, other shades joining the group from each of the houseboats, all making their way to a large central building.

As they went inside, Alice was delighted to see Ferdinand, the monkey, seated at one of the tables. She darted over, and slid into the chair next to him. "So you aren't going to be late to the tea," said Alice.

"Oh, that was yesterday," said the monkey, "this is tomorrow."

"At any rate," said Alice, "apparently we have jam this week," as she took a huge slice of bread and butter spread thickly with strawberry preserves. While munching this, she looked about her with amazement. At one end of the table was Puck, and at the other, the Mad Hatter. All of her recent acquaintances were there, and across the table was Pasteur dilating to Lord Lister on the value of laboratories to the human race, and right next to Lister were Tweedle Dum and Tweedle Dee.

On her right, Erasmus and the Unknown Soldier were discussing methods of preventing war. Said Erasmus[11], "One war springeth of another. . . . There is agreement among poisonous serpents. But unto man, there is no wild or cruel beast more hurtful than man. . . . Moreover, when the brute beasts fight, war is one for one, yea and that very short. . . . When was it ever heard that an hundred thousand brute beasts were slain at one time fighting and tearing one another? . . . Now if man will weigh, as if it were in a pair of balances, the commodities of war on the one side and the incommodities on the other side, he shall find that an unjust peace is far better than righteous war. In war, the most part of the punishment and harm falls upon them that least deserve to be punished, that is, upon husbandmen, old men, honest wives and young children. There is no tongue can tell the harm and mischief that we feel in war." The Unknown Soldier gravely nodded his head in complete assent, then said that he had written a few verses that expressed his hopes and dreams.

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[11] Erasmus, "Against War."

THE DREAM OF THE UNKNOWN SOLDIER

The filth and squalor, the lice, the stench,
The shrieking of shells, death in the trench,
To me are but memories far in the past.
Would that the World War *had* been the last!
A dream have I for the "brave new world"
Of beauty and truth, with war banners furled.

Sweet grapes, fresh milk, hot bread and roast meat,
Soft wind on cheek, damp grass on bare feet,
Sunset and skies, deep woods and swift brooks,
Friends and kind words, music and books,
Work and fatigue, achievement with zest,
Home after storm, and well-earned rest.

Ample for all, and none with too much,
With leisure for painting, 'cello, and such.
No excess of power, no surfeit of wealth,
"No one is free who rules not himself."[12]
Full scope for each, both humble and brave
With teachers of spirit from cradle to grave.

This free-will choice, each man must make
'Twixt growth of self, and selfish sake.
Beauty is near for all to enjoy,
But truth lies hidden in hard alloy.
Humane ideals, not lust for power
Must rule, if the World would truly flower.

"I'm afraid I don't quite understand," said Alice timidly. Neither did Ferdinand for he grabbed two bananas from the table and darted for the door. Alice made after him, intending not to let him get away from her this time. But she stubbed her toe on the doorsill and felt herself falling, falling, not to the floor, but into her father's arms.

"It is time to wake up, Alice," said her father. "You have had quite a nap."

"Oh—where's Ferdinand,—where's the monkey?" said Alice, rubbing her eyes. "I've had the strangest dream, all about viruses and people and everything. I must tell you all about it."

"Well, it is time to go home to supper now, and then you may continue your dreams. For tomorrow we start bright and early for the unscanned western mountains, and the distant Golden Gate.—Do you think we shall get there?"

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[12] Epictetus.

"Of course," said Alice dreamily. "But it will take a long time."

"Yes," said her father. "It will take many centuries."

EPILOGUE

"He who remembers what man is, can be discontented at nothing which happens."[13]

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[13] Epictetus. This epilogue is thought to be in harmony with the philosophy of the paper but not with the spirit of the Annual Banquet, so it was not read at the meetings.

SCIENTIFIC PROCEEDINGS

FORTIETH GENERAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

San Francisco, Calif., August 30, 31 and September 1, 1938

Headquarters: Hotel Fairmont

OFFICERS OF THE SOCIETY

President, **Paul F. Clark**, University of Wisconsin, Madison, Wis.

Past-President, **James M. Sherman**, Cornell University, Ithaca, N. Y.

Vice-President, **Arthur T. Henrici**, University of Minnesota, Minneapolis, Minn.

Secretary-Treasurer, **Ira L. Baldwin**, University of Wisconsin, Madison, Wis.

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T. D. Beckwith
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Justina H. Hill
N. P. Hudson
L. B. Jensen
Rebecca C. Lancefield
H. M. Powell
G. H. Smith
M. H. Soule
G. I. Steffen
C. Thom
A. B. Wadsworth
A. R. Ward
C. H. Werkman

Program Committee

George Packer Berry, Chairman

Edwin W. Schultz, Chairman of the Section on Medical Bacteriology, Immunology and Comparative Pathology.

Paul J. Beard, Chairman of the Section on Agricultural and Industrial Bacteriology.

Local Committee on Arrangements

Wilfred H. Manwaring, Honorary Chairman
Courtland S. Mudge, Chairman
Paul J. Beard, Vice-Chairman
Morris A. Stewart, Secretary-Treasurer

Margaret Beattie
J. R. Beach
T. D. Beckwith
C. W. Bonynge
C. V. Burke
W. V. Cruess
L. L. Daines
G. E. Davis
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E. A. Fennel
J. C. Geiger
J. E. Greaves
R. A. Greene
W. V. Halversen
B. S. Henry
L. D. Hertert
Beatrice F. Howitt
H. G. Johnstone
J. F. Kessel
L. S. McClung
H. J. Sears
E. W. Schultz
M. Silverman
J. E. Simmons
Ida M. Stevens
R. V. Stone
E. L. Sugarman
W. J. B. Terrill
C. E. ZoBell

ABSTRACTS*

Report of the Committee on Bacteriological Technic: Progress during the First Half of 1938. H. J. CONN, *Chairman;* J. H. BROWN, VICTOR BURKE, BARNETT COHEN, M. W. JENNISON, J. A. KENNEDY, AND A. J. RIKER, *Members.*

This report covers the work of hardly more than six months, for the last report of the Committee on Bacteriological Technic was presented in December, 1937. During this period the Committee has revised *Leaflets VI* and *IX*, and the *Index* of the *Manual of Methods for Pure Culture Study of Bacteria.*

Sales of the *Manual* have been increasing rapidly during the last few years; so much so that in 1937 receipts from the sales of this publication were $170 larger than any preceding year, except 1923, when the *Manual* was first issued. During the first five months of 1938, moreover, receipts have been $114 larger than during the same period of 1937. The subscription list to *Pure Culture Study of Bacteria,* the continuation service for the *Manual,* has likewise been growing during this same period.

The sales of the *Descriptive Chart* have not been increasing, and it is felt that the latest revision of the *Chart* may have made it too complicated for use in teaching. Accordingly, a preliminary draft of a new and much simplified *Chart* is now being submitted to the Society for consideration. It is intended for use in instruction only and it is planned to supplement, not to replace, the present *Chart.* Although it is not to be put on sale for some time, anyone interested may receive a copy of the suggested form by application to the Chairman of the Committee or to Dr. M. W. Jennison of the Massachusetts Institute of Technology. Comments and criticisms from anyone inspecting a preliminary copy will be welcome.

Report of the Committee on Biological Abstracts. A. P. HITCHENS, *Chairman.*

It will be remembered that *Abstracts of Bacteriology,* supported by this Society as one of its official organs for 9 years, was merged into *Biological Abstracts* in 1925. Since then, *Biological Abstracts* has been functioning continuously as a reviewing and indexing periodical for all of the biological sciences.

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* This number of the JOURNAL has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, due to restrictions of time imposed by the printing schedule.

As a result of circumstances which are too well known to need discussion here, it has become necessary for biologists themselves to finance *Biological Abstracts*, if its publication is to be continued. Toward the end of last year an emergency plan was developed which has permitted publication of this year's volume. The time has now come to discuss permanent support for *Biological Abstracts*, and it is my belief that the Society of American Bacteriologists has an opportunity to procure whatever the membership may desire in the way of an abstracting journal.

Up to the present time, it has been necessary for any subscriber to purchase each annual volume of *Biological Abstracts* in its complete state covering all the phases of biology. Some bacteriologists have felt that this was wasteful, since their interests did not extend so broadly. The present board of trustees of *Biological Abstracts* is willing to change this policy. If bacteriologists and immunologists are interested, there is a chance to have *Biological Abstracts* split into sections. Our section would cover bacteriology, immunology, mycology, etc. The price per volume of the section would not be more than $5.00 per year. On the other hand, a subscriber to such a section would receive the annual index to the entire publication.

Another possibility, which to me is even more attractive, is that our Society, through an interested committee, may practically assume the editorial management of its particular section. The editor of *Biological Abstracts*, and his associates, are so coöperative that they are willing to make any practical arrangement with the Society of American Bacteriologists for publishing such abstracts of current bacteriological literature as may be furnished to them by the committee. Furthermore, such abstracts will be published promptly in accordance with the announced policy of having in print abstracts of all biological literature readily available within 2 months of the original publication.

Many other things might be said about *Biological Abstracts* and its value to bacteriologists. An abstract journal seemed so necessary to us a few years ago that we were willing to support such a publication all by ourselves. That publication left many things to be desired, but now we have the means offered to us for doing an entirely satisfactory job. In other words, if there are in our membership individuals who care to assume responsibility for getting out the abstracts promptly, the copy will be published promptly and indexed thoroughly and carefully. In all of these matters, the editorial office of *Biological Abstracts* will meet a committee of our Society at any point and carry on from there.

It is my belief that a plan can be worked out, and that the result will be attractive to bacteriologists everywhere. It will in effect give us back our *Abstracts of Bacteriology*. Then, I believe, a sufficient number of our members will add their names to the subscription list to render effective aid to a worthy project.

I am well aware of the fact that a portion of our members feels no need for a review journal to keep in touch with developments in the biological sciences. Chiefly, such persons are in large teaching and research institutions. Such individuals would seem to have no place on a committee, whose function would be that of seeking to aid less fortunately situated members in keeping abreast with current developments. May I suggest, therefore, the appointment of a committee of persons interested in an abstracting journal, persons who feel the need of it as a research instrument and who are willing to devote time and thought to the formulation of a plan by which our Society may cooperate with the editorial board of *Biological Abstracts?*

GENERAL BACTERIOLOGY

G1. Nutrient Requirements of Butyric-Acid Butyl-Alcohol Bacteria.

R. W. Brown, H. G. Wood, and C. H. Werkman, Tuskegee Institute, Tuskegee, Ala., and Iowa Agricultural Experiment Station, Ames.

Experiments were made to determine the nutrient substances essential for the dissimilation of glucose by butyric-acid butyl-alcohol bacteria. Eight cultures, including strains of *Clostridium acetobutylicum, Clostridium butylicum, Clostridium pectinovorum, Clostridium felsineum* and *Clostridium beijerinckii,* were used. The organisms were grown in tubes containing 10 cc. of medium composed of various combinations of hydrolyzed casein, ammonium sulfate, acidic ether-soluble extract of yeast extract (Difco), vitamin B_1, tryptophane, Speakman's salts, mono- and di-basic potassium phosphate and glucose. Speakman's salts were composed of K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$. Growth was measured by the quantitative determination of glucose, before and after fermentation, using a modification of the Munson and Walker method.

In the presence of ammonium sulfate, an acidic ether-soluble extract of yeast extract (Difco) and Speakman's salts were essential for the vigorous growth of all of the organisms tested. Vitamin B_1 and tryptophane were not essential. Neither Speakman's salts minus phosphates nor the phosphates without the other inorganic salts were sufficient for growth in successive transfers.

With purified, hydrolyzed, vitamin-free casein as the source of nitrogen either the ether-soluble extract of yeast extract (Difco), Speakman's salts or phosphates alone were essential for growth in successive transfers. Speakman's salts minus the phosphates were not sufficient for continued growth. Tryptophane and vitamin B_1 were not essential.

Fermentations yielding the normal end-products from the dissimilation of glucose were obtained in both ammonium sulfate and hydrolyzed casein media when the other essential nutrients were present.

G2. The Utilization of Carbon Dioxide by the Propionic Acid Bacteria.

H. G. WOOD AND C. H. WERKMAN, Iowa Agricultural Experiment Station, Ames.

Carbon dioxide supplied in the form of $NaHCO_3$ or $CaCO_3$ is utilized by propionic acid bacteria in the fermentation of glycerol. Utilization of CO_2 is accompanied by a molar equivalent formation of succinic acid. It is suggested that succinic acid may arise by synthesis from 3- and 1-carbon compounds.

Carbon dioxide uptake has been investigated in the Warburg respirometer, using non-proliferating cell suspensions or dry cell preparations in phosphate buffer and under an atmosphere of CO_2. Dissimilation of glycerol, erythritol, adonitol, mannitol, rhamnose and xylose showed an uptake of CO_2. Glyceraldehyde, dihydroxyacetone, arabinose, galactose and glucose were fermented with formation of no or very little CO_2, but under nitrogen significant amounts were formed. The implication is that the utilization of CO_2 increases with CO_2 concentration. The uptake of CO_2 or the fermentation of glycerol is not inhibited by KCN, 0.1 per cent; arsenite, 0.01 M; malonate, 0.03 M; pyrophosphate, 0.02 M; and sodium azide, 0.002 M. However, NaF, 0.00125 M, inhibits the utilization of CO_2 in glycerol dissimilation and increases the output of CO_2 from glucose. The increased yield from glucose probably is caused by the inhibition of CO_2 uptake. Although a net decrease in CO_2 has not been obtained as yet for all substrates, it seems probable that CO_2 may be utilized in most, if not all, dissimilations under the proper conditions.

G3. *Studies on the Relationship between Synthesis and Respiration.*

C. E. CLIFTON, Stanford University, Calif.

Recent studies by the author show that the oxidation of acetate, butyrate or lactate is not carried to completion by well washed suspensions of *Escherichia coli.* The results suggest that a portion of the substrate is oxidatively assimilated by the cells and that the assimilatory process is blocked in the presence of suitable concentrations of sodium azide or 2:4 dinitrophenol, the oxidation then proceeding to completion.

These studies have been extended to include the oxidation of fumarate, succinate, glucose and a number of amino acids by well washed suspensions of *Escherichia coli* in phosphate buffer or in a saline-bicarbonate solution in equilibrium with a 5 per cent CO_2-95 per cent O_2 gas-mixture. The consumption of O_2 and the production of CO_2 were determined at 30°C. by the Warburg technic. The extent to which a given compound is oxidized has been found to vary markedly with the nature of the substrate, being approximately 75 per cent for acetate and 50 per cent for glucose. With the majority of compounds tested, it approached 100 per cent in the presence of suitable concentrations of sodium azide or 2:4 dinitrophenol.

Studies on the extent of oxidation of lactate or glucose during the growth of *Escherichia coli* in an inorganic medium gave results similar to those obtained with the washed suspensions. These, and other results which will be discussed, suggest that the respiration of heterotrophic bacteria may well be an oxidative assimilatory reaction which may be represented by the general equation:

$$\text{Substrate} + O_2 \rightarrow (CH_2O) + CO_2 + H_2O$$

This is much like the reaction which represents the assimilatory process in the green plant:

$$CO_2 + H_2O \xrightarrow[\substack{\text{radiant}\\ \text{energy}}]{} (CH_2O) + O_2$$

G4. *Occurrence of Glutathione in Microörganisms.*

T. E. MILLER AND R. W. STONE, Pennsylvania State College, State College.

Glutathione, the sulfhydryl-bearing tripeptide associated with cell respiration, was quantitatively determined in various bacteria, yeasts and molds by use of the iodate-titration method of Quensel and Wachholder. The nitroprusside test was used as a qualitative check, and in practically all of the experiments it correlated with the quantitative determination. Members of the following genera showed determinable amounts of glutathione in the cell: *Aerobacter*, *Escherichia*, *Proteus*, *Chromobacterium*, *Serratia*, *Rhizobium*, *Azotobacter*, *Lactobacillus*, *Saccharomyces*, *Torula*, *Penicillium*, *Aspergillus*, *Rhizopus*, and *Monilia*. Four species of *Clostridium* had relatively small amounts; the cocci and members of *Bacillus* showed negative or questionable quantities. In comparison with normal human blood which has been reported as containing from 34 to 47 mg. of glutathione per 100 gm. sample, *Aerobacter aerogenes* contains about 27; *Proteus vulgaris*, 29–31; *Chromobacterium violaceum*, 7–45; baker's yeast, 59; *Torula sp.*, 57; and *Monilia sitophilia*, 20–38 mg. per 100 gm. of moist organisms.

Both aerobes and anaerobes contain the tripeptide, thus there is no apparent correlation of glutathione content with anaerobiosis. The data indicate that glutathione is an intracelllar substance, since the culture medium usually contained only from 2 to 20 per cent as much of the peptide as the organisms. As glutathione is the coenzyme of methylglyoxalase, these results lend support to the theory that it is possible for methylglyoxal to act as an intermediate in most microbiological fermentations.

G5. The Effect of Certain Amino Acids and Ascorbic Acid on the Growth of Staphylococcus aureus in Casein Solutions. LIONEL FARBER, Hooper Foundation for Medical Research, University of California, San Francisco.

A study was made of the growth of a strain of *Staphylococcus aureus* in a 1 per cent casein-inorganic salt medium to which were added varying quantities of the substances to be tested. The growth was measured by determining the total nitrogen in the washed bacterial centrifugate. The following amino acids separately stimulated the development of the organism: *d*-glutamic acid, *d,l*-histidine, *d*-arginine, *l*-proline, *l*-hydroxyproline, *l*-cystine and *l*-cysteine. Ascorbic acid also had a growth-promoting effect. The increases varied with the individual substance and the concentration. Extracts of beef liver, spleen and kidney, of hog pancreas and small intestine, and of yeast elicited more marked growth-enhancing actions than any one of the above materials.

G6. Certain Factors Affecting the Growth of Yeast. NATHAN F. TRUE, LLOYD PAUL, NORMAN J. MILLER AND PAUL S. PRICKETT, Mead Johnson and Co., Evansville, Ind.

The enumeration and control of yeasts are important problems in industries other than brewing and distilling. These problems, particularly rapid methods of enumeration, are dependent on factors affecting the growth of yeast. Bacterial growth interferes with such methods and may cause confusion unless controlled.

In both liquid and solid media the following factors were found to be important: composition of nutrients in the medium, concentration of carbohydrates, hydrogen ion concentration, atmospheric oxygen, and fractions of the vitamin B complex. It appears, however, that various types of yeast are influenced more markedly by some of these factors, either singly or in combination, than are other types.

The following results are typical. Malt syrup was plated on Wort Agar (Bacto) and incubated for 4 days at room temperature to afford a basis of comparison. The yeast plate count was 20 per gm. of syrup. Various dilutions of the malt syrup ranging from 10 to 50 per cent were prepared with sterile water. In the plain malt syrup-water dilutions bacterial growth was a complication. One-tenth per cent of thiamin chloride (vitamin B_1) accelerated the growth of yeast but did not check the bacteria. Adjusting the pH to 4.7 did control the bacteria. A 30 per cent malt syrup concentration, adjusted to pH 4.7 and containing 0.1 per cent of thiamin chloride, gave typical gas production and characteristic odor of yeast fermentation in 56 hours. The diagnosis was confirmed microscopically.

In a series of tests on different malt syrups, the above method has proved to be more sensitive as well as more rapid.

G7. The Cultivation of Bacillus coli and Bacillus typhosus on Synthetic Media Containing α-Alanine, l-Cystine, l-Tryptophane and d-Threonine as Sources of Nitrogen. M. H. Soule and Helen Loomis Gehring, University of Michigan, Ann Arbor.

A basic medium was prepared as follows: doubly distilled water, 1,000 cc.; glucose, 1.0 gm.; $MgSO_4$, 0.01 gm.; $CaCl_2$, 0.01 gm.; K_2HPO_4, 1.0 gm.; KH_2PO_4, 1.0 gm. It was sterilized in the autoclave at 120°C. for 15 minutes or by passage through Berkefeld W filters. The final pH was 6.8. The organisms under consideration would not grow on this medium. Individual lots, therefore, were enriched by the addition of a single amino acid, as follows: α-alanine, 1.0 per cent; *l*-cystine, 0.038 per cent; *l*-tryptophane, 0.1 per cent and *d*-threonine, 0.1 per cent. *Bacillus coli* grew luxuriantly on the medium containing the α-alanine. *Bacillus typhosus* failed to multiply. A period of adaptation was necessary before *Bacillus coli* grew well on the medium containing *l*-cystine. *Bacillus typhosus* failed to grow initially on the cystine-containing medium, but an increase in the pH to 7.4 by the addition of K_2HPO_4 plus 0.0002 per cent of ferric chloride permitted growth. Growths of *Bacillus coli* were obtained on the medium containing *l*-tryptophane, provided ferric chloride were present, and on the pabulum containing *d*-threonine. *Bacillus typhosus* grew in the presence of *l*-tryptophane plus iron, although the addition of sodium lactate with gradual withdrawal was essential.

Several strains of *Bacillus coli* and *Bacillus typhosus* have been maintained by serial subculture at five-day intervals on the aforementioned media for over 100 serial transfers. Other amino acids have been substituted with negative results. Transfers to the usual differential media showed that the cells had not been fundamentally changed. Contrary to the reports in the literature, tryptophane did not seem to be essential to the growth of *Bacillus typhosus*.

G8. Metabolic Studies of a Non-Hemolytic Streptococcus. J. W. King, J. C. Garey and M. A. Farrell, Pennsylvania State College, State College.

A study was made of the effect of casein-acid digests, and the Dakin butyl alcohol fractions from these digests, on the growth of a non-hemolytic streptococcus. Under the conditions of this study, when the hydrolysate is fractionated according to the butyl-alcohol method of Dakin, it has been shown that the growth-stimulating properties of the hydrolysate are present exclusively in the butyl alcohol-insoluble fraction. This fraction contains the diamino and dicarboxylic amino acids.

The monoamino, monocarboxylic fraction exerts no effect on the growth of the test organism. The proline fraction, soluble in moist and dry butyl alcohol, actually inhibits growth. This inhibitory factor acts in extremely small concentrations and has not been identified. Inasmuch as the unfractionated digest does not evidence this effect, it is assumed that the process of fractionation and of purification altered this part of the digest in some way.

In an attempt to substitute various amino acids for the activating fraction, no single amino acid was found which entirely replaced the butyl-alcohol-insoluble fraction. The amino acids examined may be listed as activating, inhibitory or exerting no effect. Lysine, all monoamino, monocarboxylic acids, except alanine and α-amino valeric acid, and including the sulfur-bearing amino acids, exert no effect. Glutamic acid, histidine, arginine, alanine, and α-amino valeric acid activate. Tryptophane is the outstanding inhibitory substance. Growth was measured by photometric readings, which are believed to be more accurate than visual estimations of turbidity, Nesslerization, or micro-Kjeldahl procedures.

G9. Cultural Requirements for the Production of Black Pigments by Bacilli. FRANCIS E. CLARK AND NATHAN R. SMITH, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.

The conditions required for the blackening of media by 38 cultures of aerobic, spore-forming bacilli were studied. It was found that *Bacillus niger* produces black pigment upon protein media which contain free or metabolically available tyrosine. Some commercial peptones containing no readily available tyrosine are not blackened unless free tyrosine is added. The addition of a fermentable sugar to many protein media normally blackened by *Bacillus niger* prohibits pigmentation. *Bacillus aterrimus* blackens media containing fermentable carbohydrates, either in the presence or absence of tyrosine, but does not blacken sugar-free peptone media readily blackened by *Bacillus niger.* Carbohydrate media containing mineral nitrogen are blackened by *Bacillus aterrimus,* but not by *Bacillus niger.* Cultural differences between *Bacillus aterrimus* and *Bacillus niger* are believed sufficient to warrant their recognition as distinct species. *Bacillus tyrosinogenes* of Rusconi and *Bacillus nigrificans* were found similar in all respects to *Bacillus aterrimus,* and are believed to be synonymous with that species. *Bacillus betanigrificans* produces black coloration in the presence of iron, but not upon the media blackened readily by the species considered above. *Bacillus betanigrificans* possesses morphological and cultural differences that separate it from *Bacillus niger* and *Bacillus aterrimus.*

G10. Molds as Protein Food in Diets. C. E. SKINNER AND EUGENE MULLER, University of Minnesota, Minneapolis.

Aspergillus oryzae, Penicillium roquefortii, Penicillium flavo-glaucum, and two unidentified species of *Penicillium* were grown on media composed of inorganic salts, water, and sugar. The dried, ground mycelium was fed to rats as the sole source of protein in otherwise complete diets. The only other organic nitrogen consumed was 100 mg. of yeast-extract powder fed daily. Paired-animal experiments showed that a slow growth-rate was maintained, but that methionine was present in minimal amounts, since a statistically significant stimulation to growth was evinced when 0.25 gm. cystine or methionine, but not when any other amino acid tested, was added to 100 gm. of ration. The molds synthesized from inorganic-nitrogen complex compounds indispensable for animal growth as lysine, valine, tryptophane, histidine, phenylalanine, leucine, iso-leucine, α-amino-β-hydroxy-n-butyric acid, and small amounts of methionine. Positive Millon reactions showed tyrosine or other phenolic compounds to have been synthesized. When maize or wheat was cracked, moistened, sterilized, and inoculated with *Penicillium flavo-glaucum*, allowed to become very moldy, and dried, there was no significant difference between moldy grain as a protein source in diets and similar grain so treated but uninoculated. This was true whether or not the protein content of the moldy grain was brought to the level of the uninoculated grain by the addition of starch. The mold did not synthesize sufficient tryptophane or lysine to overcome significantly the amino acid deficiencies of these grains.

G11. Observations on Marine Anaerobes in Oval Tubes. CLAUDE E. ZOBELL AND D. Q. ANDERSON, Scripps Institution of Oceanography, University of California, La Jolla.

Thin-walled oval tubes, 6 x 14 mm. in cross-section and 380 mm. long, have proved useful for the enumeration and isolation of anaerobic bacteria, as well as for studying their metabolism and E_h requirements. The application of such tubes has revealed that sea water contains from one to nearly a hundred anaerobes per cc. Most are facultative anaerobes capable of growing over a wide E_h range. The anaerobic population of bottom deposits ranges from a few to over a million per gm. of mud. The total number of anaerobic bacteria decreases with core depth, but the proportion of strict to facultative anaerobes increases.

The ease with which oxygen can be excluded from the long narrow tubes facilitates poising the media at the desired E_h values by means of appropriate reducing or oxidizing agents. Microelectrodes have been perfected for making continuous measurements of the E_h and also the pH of different strata in the oval tubes. Most of the strict anaerobes from the sea start to multiply around E_h 0, although the optimum for their multiplication is between E_h -150 and -250 millivolts. They generally reduce the oxidation-reduction potential to about E_h -350 millivolts, although potentials as low as E_h -580 millivolts have been observed.

By using selective media, the oval tubes have proved useful for estimating the relative abundance of different physiological types of anaerobes (provided they do not produce gas). Special attention has been given to the study of lipolytic anaerobes isolated from the sea.

G12. The Presence in the Sea of Bacteria Belonging to the Genus Clostridium. MARGARET HOTCHKISS, Oceanographic Institution, Woods Hole, Mass.

The examination of marine samples for the presence of members of the genus *Clostridium* has confirmed the findings of a preliminary study reported in 1933 from the Woods Hole Oceanographic Institution. Organisms having the morphology of *Clostridium* were found in "bottom-material" and were most abundant near shore, where decomposable substances were available. Samples of water taken from near the shore also showed the organisms. Diatom "tow" afforded a site where the organisms could thrive. The organisms fermented glucose with the production of gas and of butyric acid. Mixed cultures were able to grow in a nitrogen-free medium and to fix nitrogen. The organisms have been isolated in pure culture. In morphology they resemble members of the genus *Clostridium* commonly present in soil. Growth on artificial media has been very scanty.

G13. Physiological Events during the Dissimilation of Carbohydrate by the Living Bacterial Cell. C. H. WERKMAN, Iowa Agricultural Experiment Station, Ames.

A general review will be presented of the comparative behavior of the bacterial cell with respect to its aerobic and anaerobic dissimilation of glucose. In the light of present knowledge, an attempt will be made to reconstruct the physiological events in the living bacterial cell, particularly with respect to the transformations undergone by carbohydrate. The discussion will include a consideration of dehydrogenase systems, principal and subsidiary respiration, and final transformation of the intermediates by the bacterial cell.

G14. Recent Observations on the Enzymic Hydrolysis of Fats and Other Esters. A. K. BALLS, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Lipase differs from most enzyme-systems in that the enzyme is soluble in water while the substrates usually are not. The manner of performing a fat digestion, therefore, is generally admitted to be of unusual importance. By means of a new technic a number of substances, hitherto untested or regarded as unaffected by lipase, have been rapidly split. It cannot be taken for granted that the entire scope of lipase activity is thus displayed, for the methods may still be imperfect. Nevertheless, an examination of these new data leads to the interesting assumption that the enzyme is exclusively a primary alcohol esterase. The conflict of this statement with the known digestion of triglycerides must be reconciled before it is acceptable. A study of the digestion of synthetic addition compounds of mono- and di-glycerides has so far fitted in with the explanation that the glyceride molecule undergoes rearrangement during hydrolysis. The latest developments of this study, which is still in progress, will be reported.

G15. The Proteolytic Enzymes of Microörganisms. MARVIN J. JOHNSON, University of Wisconsin, Madison.

Recent investigations from several laboratories have added considerably to existing knowledge of the proteinases and peptidases of microorganisms. Although yeasts and a few other microörganisms have been shown to produce proteinases of the papainase type, a large number of microörganisms produce proteinases, characterized by optimal activity at pH 7 and by lack of activation by reducing agents, which do not appear to belong to any previously studied proteinase group. Many bacteria produce a proteinase which splits gelatin readily, but attacks other proteins very slowly if at all. In some species, this is the only proteinase secreted.

The peptidase systems of molds are fairly similar to each other and, in some respects, to previously studied yeast and animal peptidase systems. Bacteria exhibit unusually wide generic differences in their peptidase systems. Examples may be found among the bacteria of almost any known type of peptidase system. Moreover, a number of previously unknown types of peptidases have been found in bacterial cells. Many anaerobic species produce peptidases which are activated by heavy metals and by reducing agents. A number of lactic acid bacteria contain peptidases unique in acting most rapidly in acid media. Some bacterial peptidases are able to hydrolyze unnatural optical forms of peptides, not attacked by previously studied peptidases.

G16. Studies of Respiratory Enzymes Applied to Problems of Bacterial Metabolism. P. W. WILSON, University of Wisconsin, Madison.

The technics originally developed for the study of the respiratory activities of tissue cultures have been modified for the study of bacterial reactions. These technics consist essentially in the use of non-proliferating organisms ("resting" cells) and the measure of enzymic reactions through microprocedures, *i.e.*, the use of microrespirometers, Thunberg tubes, and microchemical determinations. The chief advantages of this approach to the study of bacterial metabolism are: (1) respiratory activities are separate from those of growth; (2) "isolation" of the enzyme-systems is accomplished through the use of specific substrates and specific inhibitors; (3) data concerned with the kinetics of reaction are readily obtainable; and (4) the experiments are of brief duration, thereby eliminating differences in metabolism associated with cells in various stages of development and allowing comparison of these to be made.

Problems in bacterial metabolism which will be discussed as illustrations of the application of the use of the respiratory-enzyme technics will include: (a) the hydrogen enzymes of *Escherichia coli*—the evidence will be reviewed for the existence of three specific enzymes which are essential for the transfer and liberation of hydrogen by cultures of *Escherichia coli;* (b) the mechanism by which anaerobes secure their energy—discussion will be given of the so-called "Stickland reaction' whereby anaerobes secure energy through reactions involving two amino acids; and (c) species and strain variation among the root-nodule bacteria—a study of the characteristics of the respiratory-enzyme systems of members of the several cross-inoculation groups of the root-nodule organisms will be summarized.

G17. The Rôle of Bacterial Enzymes in Immunology. M. G. SEVAG, University of Pennsylvania School of Medicine, Philadelphia.

The phenomenon of catalysis is universal and fundamental in every living cell. It has a direct bearing on the nature and extent of immunological events. The chemical constitutions and specific activities of bacterial enzymes and antigens possess many similar characteristics. The metabolic activity of bacteria is lost as a result of inhibition, impairment or aging. Under these conditions, and in the absence of substrates, organisms begin to undergo autolysis in association with endocellular oxidative processes. Also shown is the increase of oxygen consumption during the lytic action of specific bacteriophage on bacteria. In metabolic studies *in vitro*, the normal course of bacterial enzymic activity can be completely inhibited or modified by the presence of activating or inhibiting agents. These effects under conditions of growth cause structural variations in bacteria. Similar studies on bacteria combined with specific antibody, in the absence or presence of complement, have shown altered metabolic activities. Such influences exercised repeatedly on bacteria cause fundamental changes and alter immunological behavior. Bacterial enzymes are definitely affected by such phenomena as: agglutination, bacteriolytic complement fixation, bacterial variation in the presence or absence of immune bodies, and phagocytosis in the presence of opsonins and non-specific growth- and enzyme-inhibiting agents.

G18. Commercial Production and Industrial Application of Enzymic Preparations Derived from Microörganisms. Leo Wallerstein, Wallerstein Laboratories, New York City.

The pioneering work of Takamine, Boidin, Effront and others, has enabled considerable progress to be made in the commercial production of highly potent enzymic preparations from bacteria, yeasts and molds. A description will be given of present methods for the manufacture of such preparations and certain of their industrial applications will be discussed.

Historically, the use of enzymic preparations in industry falls into several stages. At first, the effects were simply those of the enzymes secreted by the various microörganisms which happened to be present. Later, the conditions necessary for the desired results were established empirically. With the realization that the reactions were due to certain enzymes and with advancing knowledge of their specific properties, the production of the enzymes themselves naturally followed as a means for controlling the reactions.

The principles involved in the various methods of commercial manufacture will be referred to. As typical, the paper will discuss the production of invertase from yeast, of amylase and protease from *Bacillus subtilis*, and of amylases from *Aspergillus oryzae*. The characteristics of these enzymes and the equipment necessary for their large scale production will be considered. Finally, some important industrial applications will be presented of the ways in which these enzymic products are used in the textile industry, the tanning industry and the various food industries. It is suggested that there are many possibilities for profitable industrial applications of these and other enzymic preparations in new fields. Such applications await only a more general knowledge of the extent to which enzymic preparations are commercially available.

G19. Adaptive Bacterial Enzymes: The Mechanism of Their Production and Their Application in Biochemistry and Immunology. René J. Dubos, Hospital of the Rockefeller Institute for Medical Research, New York City.

Karström has classified bacterial enzymes as "constitutive" or "adaptive". The former are always produced irrespective of the composition of the culture medium, the latter appear only when the enzyme-substrate is a component of the culture medium. Different theories have been formulated to account for the production of adaptive enzymes: (a) natural selection—this theory assuming that the bacterial species under consideration always gives rise to a few cells (variants or mutants) endowed with the property of producing the specific enzyme, and that these cells are selectively favored when the proper substrate is present in the medium; (b) chemical adaptation—the production of the enzyme being due to a specific chemical interaction between the protoplasm of the cell and the substrate.

Adaptive enzymes of bacterial origin appear to exhibit a remarkable specificity. They can be used, therefore, for the investigation of certain biological problems. Two examples will be discussed: (1) the detection and estimation of creatinine in body fluids by an enzymic method; and (2) the study of the rôle of capsular polysaccharides in determining the specificity and virulence of encapsulated pneumococci.

G20. *The Variability of Bacteria with Regard to Their Production of Enzymes.* E. J. ORDAL, University of Washington, Seattle.

The subject of bacterial variation with respect to the production of enzymes will be reviewed. There exist conflicting opinions as to whether the bacterial cell can change its enzymic action by producing entirely new enzymes or by discontinuing the production of others. The evidence for the acquisition of new enzymes by bacteria, as well as for the loss of enzymes normally present, will be discussed.

The mechanism of gas production by *Escherichia coli* has been studied by a comparison of the enzymes of normal *Escherichia coli* with the enzymes of anaerogenic variants. The opinion has been expressed in recent years that hydrogen can be produced directly from glucose by means of an enzyme, "glucose hydrogenlyase", which is considered to be separate and distinct from "formic hydrogenlyase", the enzyme producing gas from formic acid. Furthermore, "formic hydrogenlyase" is considered to be separate and distinct from "formic dehydrogenase", the enzyme concerned in the activation of formic acid, and from "hydrogenase", the enzyme concerned in the activation of molecular hydrogen. The evidence obtained from this study indicates that the hydrogen gas produced from glucose by *Escherichia coli* comes from intermediately formed formic acid, and that formic dehydrogenase and hydrogenase are constituents of the enzyme liberating gas from formic acid.

G21. Variation in Certain Biochemical Characteristics of Bacteria.

W. J. NUNGESTER, University of Michigan, Ann Arbor.

Variations in certain biochemical activities of an organism of the paracolon type have been studied. This organism did not produce acid from lactose, maltose or sucrose, when grown on a solid medium. After repeated subculturing in liquid media containing these sugars, the organisms changed so as to produce acid readily when grown on agar containing the same sugar. Certain factors were studied with respect to their ability to influence this change in biochemical activity.

It was observed that a decreased oxygen tension, as found in the lower portion of a broth culture or on a solid medium under anaerobic conditions, tended to stimulate the production of acid from the carbohydrate. Dilution of the broth base had the same effect at times. Previous training of the organism to ferment lactose or maltose did not appear to shorten the time required to train it to utilize sucrose. The ability of this organism to ferment the carbohydrates in question was not retained indefinitely. The changes in fermentative action were all of a gradual nature.

G22. Physiological Youth of Bacteria as an Important Factor in Their Adaptive Formation of Enzymes.

C. P. HEGARTY, Cornell University, Ithaca, N. Y.

Tryptone broth containing a single sugar was inoculated with 10 per cent of a fully grown culture of *Streptococcus lactis* grown in the same medium. Each hour, samples were centrifuged, the sedimented cells resuspended in phosphate-buffer solutions, and the rate of fermentation of various sugars was determined by titrations at frequent intervals. All of the cells fermented glucose at once at maximal rates. A constitutive enzyme-complex forms lactic acid from glucose.

Glucose-grown cells, one hour old, showed a delay of one-half hour before acid production from sucrose became measurable. Invertase is evidently an adaptive enzyme in this organism. Two- and three-hour-old cells produced acid immediately, and the growth curve showed that these cells were just coming out of the lag phase. Logarithmic growth was established after four hours, and the delay of sucrose fermentation was one hour. Older cells required longer initiation times; eight-hour-old cells requiring three and one-half hours. Plate counts and direct Petroff-Hausser counts showed no increases in the numbers of resuspended cells during fermentation.

Essentially the same results were obtained by growing the cells on certain other sugars. The time required for adaptation varied both with the sugar on which the cells were grown and the sugar fermented. In each case the physiologically young cells, just coming out of the lag phase, adapted themselves to new carbohydrates much more rapidly than cells in the logarithmic phase of growth.

G23. A Comparison of the Biological Activities of Certain Slow Lactose-Fermenting Bacteria and of Rapidly Fermenting Strains Derived from Them. E. R. Hitchner, E. A. Donagan and S. Alpert, University of Maine, Orono.

A comparison has been made of the morphological, cultural, biochemical, and serological characteristics of 15 strains of coliform bacteria, which exhibited delayed lactose utilization, and of rapidly fermenting strains derived from each of them. Most of the strains, which were obtained from several investigators, were isolated from abnormal stools. The rapidly fermenting variants were obtained by several serial passages through lactose broth. Two strains failed to produce rapidly fermenting variants by these methods.

On the basis of their biochemical and serological properties, the organisms were divided into 9 groups. No major differences in the morphological, cultural, and serological characteristics were observed between the slow lactose-fermenting strains and the rapidly fermenting strains derived from each. Five strains which fermented lactose slowly (6 to 9 days) likewise showed a delayed utilization of melibiose. However, coincident with the rapid utilization of lactose, each of these strains acquired the property of fermenting melibiose in 1 day. The rapid lactose-fermenting strains derived from 4 of the cultures fermented raffinose in 1 day, whereas the slow lactose-fermenting parent strains failed to utilize this carbohydrate in 30 days.

These data support the observations of other investigators that microorganisms whose fermentative activity in the rate of utilization of a certain carbohydrate has been markedly accelerated, through suitable "training", will frequently exhibit a similar acceleration in the fermentation of other carbohydrates, toward which they were originally either slightly or completely inactive.

G24. The Sensitivity of Propionibacterium pentosaceum to Sodium Fluoride as a Function of the Conditions of Growth. W. P. Wiggert and C. H. Werkman, Iowa State College, Ames.

It has been found possible to obtain two types of cells of *Propioni-*

bacterium pentosaccum with respect to sodium fluoride sensitivity. At slightly acid reactions, the glucose dissimilation by cells grown in the absence of sodium fluoride is completely inhibited by this poison, while the dissimilation by cells grown in the presence of the fluoride is not affected. The fermentation of phosphoglyceric acid, a proposed intermediate isolated from a glucose fermentation and inhibited in its breakdown by sodium fluoride, is greater with the former type than with the latter. At higher hydrogen ion concentrations, the cells grown in sodium fluoride are sensitive to this poison.

The phenomena observed may be explained by enzymic differences between the two types of cells, differences in accessibility of fluoride and substrate to the enzymes, or possibly by differences in both accessibility and enzymic character. Evidence for each possibility will be considered and the implications from the standpoint of intermediate metabolism will be discussed.

G25. Dissimilation of Citric Acid by Streptococcus paracitrovorus. C. R. Brewer and C. H. Werkman, Iowa Agricultural Experiment Station, Ames.

The dissimilation of citric acid by *Streptococcus paracitrovorus* was investigated by a chemical analysis of the products and by the Warburg respirometer technic. The organism does not readily attack citrate in a yeast-extract medium, but actively ferments glucose in a similar medium. When citrate and glucose are present together, the fermentation is enhanced and the citrate is rapidly dissimilated.

The presence of small amounts of sugar catalyses the breakdown of citrate, both aerobically and anaerobically. The catalytic effect is not due to the activity of the Krebs citric cycle, because it proceeds anaerobically and is insensitive to arsenite. The addition to citrate of acetylmethylcarbinol or fumarate as hydrogen-transfer agents does not produce stimulation comparable to the addition of glucose.

Streptococcus paracitrovorus does not exhibit oxygen consumption during aerobic dissimilation of citrate, glucose, or citrate plus glucose, as measured by manometric methods. Aerobic dissimilation is insensitive to arsenite.

G26. Enzyme-Systems in Nodules of Leguminous Plants. D. W. Thorne and Robert H. Burris, University of Wisconsin, Madison.

A technic has been developed for the study of fresh tissue from legume-root-nodules. After separating the enclosed bacteria from the remainder of the nodule tissue, it was found that the enzymic activity of the organisms greatly exceeded that of the surrounding material.

A comparison of the activity of the enzyme-systems of nodular material with that of non-proliferating cells of related species of *Rhizobium* showed a similar action on several substrates including glucose, arabinose, sodium fumarate, sodium succinate and sodium pyruvate. The Q_{O_2} values were considerably greater, however, in the case of the artificially cultured bacterial suspensions. The bacterial suspensions appeared to be more sensitive to some inhibitors than were the related nodular materials. Such differences were particularly evident with respect to fluoride and, to a somewhat lesser degree, to cyanide. In no instance was there any apparent sensitivity to carbon monoxide. The present status of this investigation indicates that the enzymic activities of nodules of various leguminous plants may differ considerably.

G27. Respiratory Enzymes of the Root-Nodule Bacteria. ROBERT H. BURRIS AND D. W. THORNE, University of Wisconsin, Madison.

Good and poor strains of *Rhizobium* from four cross-inoculation groups have been tested for the response of their respiratory activity to changes in pH, temperature and pressure of oxygen. The effect of a variety of inhibitors has been studied. On the whole the bacteria have been very similar, the only marked differences being that the soybean organisms have a higher pH optimum than the other species, and that the clover organisms require a higher oxygen tension than the alfalfa and soybean organisms for maximal respiration.

G28. The Production of Rennin by Pathogenic Streptococci. COSTANTINO GORINI, Milan, Italy.

In 1932, I proposed the milk-on-agar-culture method for detecting the production of rennin by microbes (*Schizomycetes*, *Actinomycetaceae* and *Saccharomycetaceae*) which fail to show it when inoculated directly into milk (*Bacillus typhi*, *Bacillus morgani*, *Bacillus lipolyticum*, *Streptococcus equi*, *Streptococcus equinus*, *Actinomyces aureus*, *Actinomyces asteroides*, *Saccharomyces cerevisiae*, *Saccharomyces lactis*, etc.). The method is described in the "Manual of Methods for Pure Culture Study of Bacteria".

The method has been applied to 168 strains of streptococci derived from pathologic sources. In ordinary milk cultures, these organisms were considered to be negative or quite irregular in respect to the production of rennin. Among the strains, 36 were *Streptococcus equi;* 8, *Streptococcus equinus;* 39, *Streptococcus mastitidis;* 9, *Streptococcus scarlatinae;* 7, *Streptococcus epidemicus;* 5, *Streptococcus cardioarthritidis;* 2, *Streptococcus morbilli;* 2, *Streptococcus viridans;* 1, *Streptococcus erysipelatis;* 1, *Streptococcus hemolyticus;* most of the others were pyogenic streptococci. All of these strains regularly curdled the milk-on-agar cultures, almost without the appearance of acid.

Errors in this rennin test arise from at least three sources: (1) the use of unsuitable milk; (2) failure to observe the results at the proper time; and (3) failure to recognize a minute coagulum. If this test be properly carried out and interpreted, it is useful for recognizing pathogenic streptococci in conjunction with other suitable procedures.

G29. A Comparison of Anaerobic Methods. NORMAN J. MILLER, ORVILLE W. GARRETT AND PAUL S. PRICKETT, Mead Johnson and Co., Evansville, Ind.

Many procedures have been proposed for the cultivation of anaerobic bacteria. Some are complicated or require expensive equipment. Simple methods are needed not only for isolation and qualitative studies, but also for quantitative work, especially in laboratories limited in equipment and resources. Three of the simplest methods described in the literature have been compared with each other and with a modification of another previously described simple technic. One of the first three methods was eliminated because of the breakage of glassware encountered. Thus, the three methods finally compared were: Weiss and Spaulding's, Spray's, and a modified deep agar shake. This last procedure, although long recognized as valuable, has been restricted largely to isolative and qualitative technics, doubtless as a result of difficulty in making accurate counts in a round tube.

The use of an oval test tube, the two broader sides of which are approximately parallel, enabled us to develop a more accurate deep agar shake method suitable for quantitative studies. As purchased, the oval test tube is difficult to plug, the agar has a tendency to wet the plug on agitation following inoculation, and a large surface is exposed for the absorption of oxygen. To overcome these drawbacks, we secured oval test tubes that have a round lip and neck. Furthermore, following ZoBell, we cover the inoculated agar with a plug of glucose-peptone-methylene blue agar to serve as an oxygen seal and as an indicator of anaerobiosis. In our hands Weiss and Spaulding's method has proven to be the most accurate and satisfactory, with the modified deep agar shake a close second.

G30. *A Simple Technic for Concentrating Tubercle Bacilli in Sputum.* N. P. SULLIVAN AND H. J. SEARS, University of Oregon Medical School, Portland.

The method consists in adding to sputum a small quantity of the enzyme papain in powdered form, and incubating the mixture for a few minutes. No further treatment is necessary before centrifuging. The concentration achieved is equal to that obtained by commonly used technics. The disintegration of the mucoid material is rapid and complete, and the sediment obtained spreads easily and adheres well to the slide. The bacteria and other cellular material are unaffected by the process. Cultivation may be obtained by treatment of the sediment by the usual methods.

G31. *The Evaluation of a Group of Germicides by the Tissue-Culture Technic.* A. J. SALLE, University of California, Berkeley.

It is customary to rate disinfectants on the basis of their phenol coefficients. This may be justifiable when the germicides are to be employed on external skin surfaces or for the sterilization of non-living material. However, when the disinfectants are administered internally or are used on mucous surfaces, the method has serious drawbacks. It is believed that a more accurate method of rating germicides for clinical use can be achieved by testing them for their effect on the growth of living tissue as well as for their ability to kill bacteria. A number, known as the "toxicity index", has been determined. It may be defined as the ratio of the highest dilution of disinfectant showing no growth of tissue in 10 minutes to the highest dilution required to kill the test-organism in the same period of time. Theoretically, the smaller the index the more nearly perfect the germicide.

The toxicity indices of a number of compounds were as follows when *Staphylococcus aureus* was used as the test-organism: iodine, 0.2; Hexylresorcinol, 0.9; silver lactate, 0.9; *p*-hydroxyphenyl-n-amyl sulfide, 1.0; silver citrate, 1.0; *o*-n-hexyl-phenol, 1.1; *p*-hydroxydiphenyl sulfide, 1.3; Metaphen, 1.5; silver protein strong, U.S.P., 1.7; silver nitrate, 1.8; phenol, 2.0; silver protein mild, U.S.P., 2.5+; Mercurochrome, 7.2+; Merthiolate, 169+.

When *Eberthella typhosa* was used, the following were the values obtained: silver nitrate, 0.11; silver citrate, 0.14; silver protein strong, U.S.P., 0.14; silver lactate, 0.15; iodine, 0.2; Metaphen, 0.4; silver protein mild, U.S.P., 0.43; Mercurochrome, 0.6; Hexylresorcinol, 0.8; *p*-hydroxydiphenyl sulfide, 1.0; *p*-hydroxyphenyl-n-amyl sulfide, 1.0; *o*-n-hexyl-phenol, 1.1; phenol, 1.2; Merthiolate, 1.6.

G32. *A Method for the Bacteriologic Testing of Chemical Solutions Used for the "Cold Sterilization" of Surgical Instruments.* George F. Reddish and Ella M. Burlingame, Lambert Pharmacal Co., St. Louis, Mo.

The present method of the Food and Drug Administration for testing "Preparations for Surgical Instruments" is satisfactory for ascertaining the disinfecting power of such solutions, but not for determining complete sterilization. There is need for a standardized procedure for testing such preparations for their sterilizing ability.

The average number of aerobic spores found on surgical, dental, and veterinary instruments after use in practice, and on contaminated razor blades, was found to be 2, the maximal number found on any instrument being 9. Based on these findings, the following method is suggested: Spread on Gillette-type razor blades 100 times the maximal number of spores found on contaminated instruments (approximately 1000), using spores of *Bacillus anthracis* from a five-day culture grown at 20°C. on nutrient agar of the following composition: 1 per cent Armour's peptone, 0.5 per cent Liebig's beef extract (Lemco), 0.5 per cent NaCl in distilled water, adjusted to pH 7.4. Allow to dry at 37°C. for 1 hour and then expose to the action of the solution being tested for 10, 20, and 30 minutes at 20°C. At the end of these periods, wash the blades in 20 cc. of sterile water for 1 minute and then transfer to 20 cc. of sterile broth of the above composition, adjusted to pH 6.8. Incubate for 3 days at 37°C. A satisfactory sterilizing solution should kill the spores of *Bacillus anthracis* under these conditions within 20 minutes. Practical tests with sterilizing solutions of known merit have shown this method to be satisfactory.

G33. *Verification of the Results Secured by the Manometric Method of Evaluation of Germicides.* J. Bronfenbrenner, A. D. Hershey and J. A. Doubly, Washington University School of Medicine, St. Louis, Mo.

Recently we suggested a method for evaluation of chemotherapeutic agents based on the estimation of the depressant effect on the rate of oxygen consumption by bacteria and by tissue suspensions, respectively, in glucose-succinate buffer. The present studies were undertaken with a view of correlating the data secured with those obtained by the usual methods for evaluation of germicides and by the animal test for evaluation of the toxic properties of a few representative disinfectants, phenol, Hexylresorcinol, iodine and 4 mercurial compounds.

We found that the difference in the intensity of depressing action of different germicides was only slightly greater than the possible error due to variation in the manometric measurements themselves, which may involve an error of ± 5 per cent. The time interval chosen for the final reading was of little importance, since practically the full effect of the disinfectants was manifested within 5 or 10 minutes of exposure and little change in the oxygen uptake took place beyond that time.

The analogous values (± 90 per cent of inhibition) for the effect of disinfectants on liver cells were taken as a measure of toxicity and were verified by the experiments *in vivo*. Obviously, direct comparison between the dilutions effective *in vivo* and *in vitro* would be meaningless, since the findings *in vivo* vary according to species, age of animals, route of injection of germicide, etc. Compared on the basis of relative toxicity, however, the results obtained showed remarkably good agreements in all cases except in that of phenol.

G34. A Multiple-Compartment Petri Dish. HAROLD LEON FRUITMAN, San Francisco Water Department Laboratory, San Francisco, Calif.

In the bacteriological examination of 18,000 standard water samples consisting of 5 tubes each, 36 per cent required streaking for confirmation, entailing much labor, a large stock of dishes, and considerable media. Overgrowth by spreaders and diffusion of metabolic products (metallic sheen) were often encountered when several tubes were streaked upon the same plate. Wax markings became illegible and labels were a nuisance. Accordingly, a multiple-compartment Petri dish was designed from Pyrex glass.

The new dish is separated into 5 compartments by rounded baffles (radial spokes) which prevent spreading, diffusion, and media skidding. Maximal streaking surface and ease of cleaning are obtained by the use of sloping floors and rounded fillets. The same amount of time is required to pour agar plates with the new as with the ordinary Petri dish with which it is interchangeable. The outer lower circumference of the dish is sand-blasted for penciled history. An annular ring limits the level of the media to 3 mm. which provides a firm streaking surface and requires but 10 cc. of media for the entire plate. On the bottom are impressed clockwise indices. On the rim are coördinated markings. The first compartment has a quick reference point.

The multiple-compartment Petri dish has many practical uses. It saves media and aids in developing a good streaking technic in classwork. During epidemics, the number, bulk, and expense of Petri dishes and media can be curtailed considerably.

G35. The Advantages of Tungsten Wire for Streaking-Needles. HAROLD LEON FRUITMAN, San Francisco Water Department Laboratory, San Francisco, Calif.

That tungsten wire is the most suitable material from which to make streaking-needles is the conclusion of a four-year study. It possesses the desired qualities of high heat-conductivity and transfer, maximal resistance to oxidation and fatigue, and proper rigidity and elasticity. Over 30,000 tubes during the period of four years have been streaked with a tungsten wire which is only just beginning to show fatigue.

C.P., cleaned and annealed, tungsten wire (General Electric Co.) 22 gauge, is cut to desired size, and held lengthwise in the hot part of a wing-tip flame (600° 1100°C.) until incandescent, then quickly plunged into a saturated solution of sodium chloride; a yellow oxide forms. The wire is repeatedly heated to incandescence until all the oxide and rough flakes are burned off, then plunged into salt solution. The wire takes on a permanently shiny black surface. The wire can be bent into shape either before or after processing, but it should be bent in slow stages while hot.

To streak a set of 5 tubes with a wire made of tungsten required 57 seconds; of platinum, 111 seconds; of nichrome, stainless steel, and chromel, 108 seconds. To become incandescent, a wire of tungsten required 1.4 seconds; of nichrome, 2 seconds; of platinum, stainless steel, and chromel, 3 seconds. When wires were flamed for 3 seconds (wing top used), the cooling times (no steam or sizzling when thrust into water at 30°C.) were: tungsten, 6 seconds; platinum, 17 seconds; nichrome and chromel, 14 seconds; stainless steel, 12 seconds.

G36. A New Type of Needle-Holder. HAROLD LEON FRUITMAN, San Francisco Water Department Laboratory, San Francisco, Calif.

Anyone who does much streaking has noticed a good deal of fatigue of the hand. There has been little change in the design of holders for streaking-needles since the inception of bacteriology. Accordingly, a new instrument has been designed. It is made of aluminum or its alloys, which were chosen after careful consideration of heat conductivity and weight. The design is functional. The needle-holder is as comfortable in use as a favorite pen; it weighs but 20 gm.

The holder is constructed as follows. A rod, 3/8″ in diameter and 7″ in length, is tapered gradually from the nose to a diameter of 1/8″ at the rear tip. A 5/32″ hole is drilled into the rod for half its length. The nose is threaded to receive a two-piece hollow clamp, which consists of a four-prong interlocking jaw-piece and a circular contractor nut. The jaw-piece is hollow to allow for an adjustable needle length, and the rear end is slotted to facilitate the removal of the clamp. The holder is machined at 1″ intervals to provide a series of encircling concave finger grips. This allows 4 positions, from a close tight pencil hold to a sketch-brush position. Each position has 3 individual grooves (for thumb and fingers) that are ribbed or knurled. Thus, a firm grip is afforded with a minimum of effort. The rear tip is handy for plugging cotton.

G37. Observations on Supersonic Vibration. Paul J. Beard and W. F Gantvoort, Stanford University, Calif.

A device for cooling suspensions undergoing supersonic vibration is described. A possible explanation of the heating effect is advanced. Preliminary experiments indicate that the killing time is related to the volume being vibrated, to the numbers of organisms per unit of volume, to the shape and to the size of the organisms.

Rods were killed more quickly than cocci, large rods more quickly than small rods. All of the organisms in a 30 cc. suspension of *Escherichia coli* containing 3 million organisms per cc. were killed in 40 minutes, while a suspension containing 4.5 million per cc. in a volume of 15 cc. required only 20 minutes for complete killing. A 30 cc. suspension with a density of 20 million per cc. showed 4,000 survivors per cc. after 80 minutes.

G38. Variation of Diphtheria Bacilli: Correlation of Certain Properties with Colonial Form. Harry E. Morton, University of Pennsylvania School of Medicine, Philadelphia.

Six years after the original description of the diphtheria bacillus, variations in the manner of growth were reported. Thus, numerous authors, while working with a variety of strains under different conditions, have recorded many types of variation. These findings must be inventoried, if successful attempts to correlate certain properties with colonial form are to be made, or if an orderly trend within the species is to be disclosed.

Laboratory and freshly isolated strains which have been studied by the author yielded smooth (S), intermediate (SR), rough (R) and dwarf (D) colonial forms. The different forms were isolated directly from clinical sources and occurred spontaneously in cultures. They were also produced by aging, by changes in the pH of the medium and by the addition of LiCl. The identity of the variants was carefully checked by means of tinctorial, biochemical and immunological reactions. Transformation of a given strain from one colonial type to another was usually accompanied by a change in the degree to which a given reaction was produced, rather than by the loss of certain reactions or the acquisition of new ones. A study of the colonial types showed that what heretofore have been described as discrepancies in the behavior of the diphtheria bacillus are really the normal variations within the species.

G39. A New Bacterial Species Isolated from the Chuckawalla (Sauromalus varius). L. F. CONTI AND JAMES H. CROWLEY, Zoological Research Hospital, San Diego, Calif.

Twenty-five chuckawallas (*Sauromalus varius*), originating on the San Estaban Islands, Gulf of California, were received by the San Diego Zoological Society on April 7, 1937. Tumor-like growths in the inferior cervical regions were noted in the majority of the specimens.

The surgical removal of one well-defined growth followed by bacteriological studies revealed a definite organism in pure culture. This organism differs in several respects from *Serratia anolium* which was isolated and described by F. Duran-Reynals and H. J. Clausen from similar lesions in another species of reptile.

The organism which we found associated with tumor-like growths in chuckawallas is a pleomorphic, Gram-negative bacterium, which actively liquefies gelatin, produces ammonia, reduces nitrates, coagulates and peptonizes milk, and produces a definite yellowish-green fluorescent pigment giving a faint diffuse coloration to the medium. It is motile by peritrichous flagella. Several sugars are attacked with the production of acid and sometimes of gas.

The bacterium has been found in pure culture in every tumor-like growth of living or dead chuckawallas of this species studied by our staff.

The organism was pathogenic to certain cold-blooded animals, but in moderate doses was non-pathogenic to guinea pigs. A soluble toxin was produced. No evidence has been found that the disease is fatal in nature.

Pigment-production studies may permit this organism to be classified in the family *Bacteriaceae*, tribe *Chromobactericae*, genus *Bacterium*. Hence, this organism is described as a new species: *Bacterium sauromali*.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

M1. The Soluble Malarial Antigen in the Serum of Monkeys Infected with Plasmodium knowlesi. MONROE D. EATON, International Health Division Laboratories, Rockefeller Foundation, New York City.

A soluble antigen is found in the serum of monkeys during the acute stages of the malarial infection caused by *Plasmodium knowlesi*. The antigen disappears from the serum after the acute infection subsides, and sometimes reappears during relapses. It gives specific complement fixation with immune monkey sera and, when injected intravenously into normal monkeys, causes the production of complement-fixing antibodies for malarial parasites without producing infection. Monkeys immunized with serum antigen are not refractory to malarial infection and possess a degree of relative immunity considerably less than that of animals with chronic infection.

The presence of the antigen in the serum is not attributable to extraction of the parasites *in vitro* after collection of the blood, nor to the fragmentation of parasites in the circulation. The antigen is regularly present regardless of the method of obtaining serum. It is not sedimented by ordinary centrifugation and is only partially thrown down at 27,000 r.p.m. for 2 hours. After fractionation with ammonium sulfate, most of the antigen is found in the albumin fraction of the serum, but a part is apparently adsorbed to the globulin precipitable by carbon dioxide. It is labile to heat (70°C.), acids, and alkalis.

An antigen similar to that found in the serum can be extracted by saline from ground parasitized red blood-cells. Antigens from parasitized cells and from serum show differences in their ability to fix complement with various immune sera, indicating that a second antigen not found in the serum is present in the parasitized red cells.

M2. Immunological Relationships of Polysaccharides of Organisms of the Eberthella-Salmonella Group. T. D. BECKWITH AND H. R. MORGAN, University of California, Los Angeles.

The experiments attempted to determine the immunological relationships of polysaccharides prepared from cultures of *Eberthella typhosa, Salmonella paratyphi-A, Salmonella paratyphi-B, Salmonella paratyphi-C, Salmonella aertrycke, Salmonella enteritidis, Salmonella pullorum* and *Salmonella suipestifer.*

Cultures were transferred on three successive days and were then streaked on 1 per cent glucose nutrient agar. Incubation followed at 20°C. or lower in order to obtain growth of a mucoid character and thus a higher content of polysaccharide. Rabbits were immunized against each organism with antigens of this material. The sera of animals immunized with *Salmonella pullorum* showed no agglutinating nor precipitating activity for the homologous antigens. For the precipitation tests, protein-free polysaccharides were prepared from cultures of each organism by the method of Heidelberger, Kendall and Scherp.

Using the antiserum prepared against each organism, agglutination and precipitation tests were conducted with each of the antigens. The cross-reactions between the various organisms in the agglutination and precipitation tests were in general parallel. A marked immunological relationship was indicated in the case of (1) *Eberthella typhosa, Salmonella enteritidis* and *Salmonella pullorum* and (2) *Salmonella paratyphi-B* and *Salmonella aertrycke.* These cross-reactions were consistent with the classification of the *Eberthella-Salmonella* group of organisms based on the somatic antigens of White and Kaufman. It is suggested that these polysaccharides represent at least a portion of the surface antigens of the organisms considered.

M3. Certain Newer Aspects of the Antigenic Composition of Hemolytic Streptococci. STUART MUDD, University of Pennsylvania, Philadelphia.

An immunizing substance has been isolated and identified as the type-specific agglutinogen upon which Griffith's classification of streptococcal types is based. This so-called labile antigen has been shown to be a nucleoprotein, and has been split into protein and nucleic acid fractions, each possessing serologic activity (Sevag, Lackman and Smolens, J. Biol. Chem., in press). Labile antigen, on injection into rabbits, produces antibodies without any injury to the animals detectable by histological examination. These antibodies give precipitation reactions to high titer with labile antigens, but have given agglutination only to comparatively low titer when tested with the whole streptococcus. As prepared now, these antigens are not labile to heat and storage, as originally described. Whether this discrepancy has resulted from the presence of enzymatic or other impurities in the earlier preparations, or from other factors, we do not at present know.

Certain nucleic acids isolated by Sevag from the cellular residue of disintegrated streptococci have precipitated specifically with antisera within Group A, but not with antisera within other Lancefield groups. Nucleic acids may evidently be considered as a new class of specific, serologically active substances.

M4. Antigenicity of Strepto-Fibrinolysin for Rhesus Monkeys. R. R. Madison and W. H. Manwaring, Stanford University, Calif.

It was shown by Van Deventer and confirmed by us that the anti-human fibrinolytic enzyme formed or secreted by *Streptococcus hemolyticus* is not antigenic for rabbits, regardless of the dosage, the route or the number of injections. Since this enzyme does not lyse rabbit fibrin, it seemed advisable to test its antigenicity for monkeys. Seven rhesus monkeys, therefore, were given 3 subcutaneous injections of 2 cc. of streptococcal filtrate (1,500 fibrinolytic units) at intervals of 4 days. Fourteen and 21 days after the last injection, these animals were bled and their antifibrinolytic titers determined by our routine technic. The average titers for this group were 3,200 and 5,000 antifibrinolytic units on the 14th and 21st days, respectively.

We conclude from these preliminary data that streptococcal fibrinolysin causes antibody formation in rhesus monkeys. This observation suggests that the lytic enzyme may exert some definite stimulating or toxic effect upon the monkey tissue. This action is not demonstrable in rabbits. This tissue-specific irritation may be the necessary stimulation to the specific production of antibodies.

M5. An Experimental Study of the Treatment of Pneumococcal Toxemia with a Special Carbon Preparation. George E. Rockwell, 2500 Melrose Avenue, Cincinnati, O.

The intravenous injection of carbon is not new. Since Conklin introduced the procedure, Saint-Jacques and others have used it extensively. Although no highly potent pneumococcal toxin has been made, lobar pneumonia in man presents the clinical manifestations of a profound toxemia. The toxin used in the present animal experiments was made by the method of Parker (anaerobic autolysis) or by that of Dick and Boor. The carbon used was animal charcoal dispersed in a saline-glucose solution. The size, the charge on the surface and the volatile content of the particles was controlled. The preparations were standardized for their ability to absorb pneumococcal toxin.

Mice which had been given lethal doses of the pneumococcal toxin could be saved by the intravenous administration of 1 mg. of carbon, even though the carbon injection was delayed by as much as 4 hours after the injection of the toxin.

Sixteen cases of pneumonia were treated with intravenous injections of animal charcoal in conjunction with antipneumococcal serum or (when that was not possible) with sulfanilamide. Only one case died—a man over seventy years of age who developed acute cardiac failure. The number of cases of pneumonia in our series is too small for statistical analysis. The good results, however, suggest that charcoal may be a valuable adjunct in the therapy of pneumonia. It should be used in conjunction with antiserum.

M6. The Production of Immunity to Experimental Pneumococcal Infection with an Artificial Antigen. WALTHER F. GOEBEL, Hospital of the Rockefeller Institute for Medical Research, New York City.

Artificial carbohydrate-protein antigens, prepared by combining the diazonium derivatives of the *p*-aminobenzyl glycosides of the disaccharide cellobiose and its uronic acid derivative, cellobiuronic acid, with the globulin of horse serum, give rise in rabbits to antibodies which in each instance are directed specifically toward the carbohydrate radical of the azo-protein antigen.

The sera of rabbits immunized with the conjugated cellobiuronic acid antigen agglutinate Type III pneumococci in high dilution. When mixed with cellobiuronic acid antiserum, the microörganisms show the typical Neufeld "quellung" phenomenon. Rabbits immunized with the artificial antigen develop increased active resistance to dermal infections with Type III pneumococci, and their sera confer a high order of passive immunity on mice against experimental infection with virulent pneumococci, not only with Type III organisms, but with Type II and VIII pneumococci as well. Rabbits immunized with the antigen containing cellobiose, on the other hand, are neither resistant to pneumococcal infection nor do their sera confer passive immunity on mice.

The chemical constitution of the cellobioside and cellobiuronide used in preparing the two antigens is identical, save for the grouping occupying the twelfth position in each derivative. In the disaccharide this grouping is a primary alcohol ($-CH_2OH$), whereas a carboxyl group ($-COOH$) occupies this position in the aldobionic acid. This slight alteration in chemical structure suffices, however, to confer upon the synthetic antigen containing cellobiuronic acid an important immunochemical property, namely the capacity to incite both active and passive immunity against pneumococcal infections in experimental animals.

M7. The Penetration of Antipneumococcal Serum into the Pneumonic Lesion in Rats Following Serum Therapy. ALICE H. KEMPF AND W. J. NUNGESTER, University of Michigan, Ann Arbor.

As a result of previously reported studies on the distribution of pneumococci in rats with experimental lobar pneumonia, it was felt that antipneumococcal serum, following intravenous injection, either did not reach the organisms present in the lungs or for some reason was ineffective there. One possibility was that the larger vessels or the capillaries were not patent; another, that the capillaries were impermeable to the serum. By the use of thorium dioxide (a radio-opaque substance), India ink and trypan blue, the patency of the arteries, arterioles and capillaries, and the permeability of the capillaries were studied. Various serological tests were performed to determine the presence of antiserum in the pneumonic lesion of rats which had been injected with large amounts of antipneumococcal rabbit serum. Animal-inoculation experiments were also done. In general, it was found impossible to demonstrate antibodies in the pneumonic lesion following the administration of large doses of antiserum.

M8. Immunization of Experimental Animals with a Soluble Antigen Extracted from Avirulent Pneumococci. RENÉ J. DUBOS, Hospital of the Rockefeller Institute for Medical Research, New York City.

Pneumococci killed with acetic acid, then extracted at pH 7.0, release in solution a fraction which is insoluble at acid reactions. The sera of rabbits, immunized intradermally with this fraction prepared from an R strain derived from Type II pneumococci, protect mice against infection with large doses of virulent pneumococci. When the immunizing antigen is used in relatively small amounts, the protective antibodies exhibit type specificity, for they afford protection only to mice inoculated with virulent Type II pneumococci. On the contrary, when larger amounts of antigen are used, the sera also protect mice against pneumococci of other types. This heterologous protection, however, is never as high, nor as constant, as that obtained against pneumococci of the homologous type.

It is evident that the antigen described above differs from the classical capsular-polysaccharide antigen since (1) it can be prepared from avirulent R cells, (2) it is effective in the rabbit when injected by the intradermal route, and (3) the protective antibodies to which it gives rise do not precipitate the capsular polysaccharide.

M9. The Use of the Skin Test with Type-Specific Polysaccharides in Controlling Serum Dosage in Pneumococcal Pneumonia. Colin M. MacLeod, K. Goodner, P. B. Beeson and C. L. Hoagland, Hospital of the Rockefeller Institute for Medical Research, New York City.

The skin test with the homologous specific polysaccharides in controlling the dosage of serum has been employed as a guide in the treatment of 104 patients with pneumonia due to pneumococci of Types I, II, III, V, VII and VIII. The test has been found applicable to the control of the dosage of antipneumococcal serum whether derived from the horse or the rabbit.

Thirteen patients showed a positive skin test before the administration of specific antibody and at a time when the disease was advancing. In this group of patients the test could not be used as a guide to therapy. Ninety-one patients showed a negative reaction before serum was given. In all but one, the test was used to control the dosage of serum, therapy being stopped upon the development of a positive test.

It is concluded that the skin test is a valuable adjunct in the serum therapy of pneumonia provided adequate criteria are maintained for its interpretation.

M10. Some Properties of the Type-Specific Proteins of Antipneumococcal Sera. Kenneth Goodner, Hospital of the Rockefeller Institute for Medical Research, New York City.

It now appears that several different serum proteins may have the properties of antibodies; indeed, it seems certain that in some immune sera one antigen may react with more than one variety of antibody protein. Moreover, within any one class of serum proteins, there appear to be antibodies having varying affinities for a single antigen.

An antibody protein having the properties of a euglobulin has been separated from Type I antipneumococcal horse serum. The addition of increasing amounts of acetyl-polysaccharide to this antibody solution gives results in terms of antibody precipitated which do not form a curve such as that obtained with whole serum, but which give a figure of ascending straight lines, three phases being clearly outlined. That these phases may be related to determinate combining groups of the antigen is shown by the fact that the deacetylated polysaccharide gives a figure showing only two ascending phases.

Simple results are also obtained with whole serum and an antigen containing galacturonic acid. It has been shown that this antigen reacts only with an antibody protein which has the properties of pseudoglobulin.

M11. Precise Evaluation of Therapeutic Antipneumococcal Sera. K. GOODNER, C. M. MACLEOD, P. B. BEESON, AND C. L. HOAGLAND, Hospital of the Rockefeller Institute for Medical Research, New York City.

The generally accepted evaluation of antipneumococcal sera is based on the mouse protection system. This can be carried out rather accurately for any one serum of any one type, provided an arbitrary value be assigned to a reference serum and provided enough mice be used for each test. There are two major objections to this system: (1) there is no reference of potency from serum of one type to another; (2) the test is quite expensive, if it be carried out in such a way as to give precise results.

The solution of the problem appears to be the immuno-chemical precipitation method of Heidelberger, Sia and Kendall, as hereinafter qualified. Each mg. of antibody protein in antipneumococcal rabbit serum (Type I) is equivalent to 185 units. Horse sera fall into two groups. The sera of group A have the value of 85 units per mg. of antibody protein, while those of group B have the value of 135 units per mg. These groups can be readily distinguished by the use of non-specific antigens, such as sulfanilic acid combined to a protein, or by differential precipitation at various temperatures. The sera of group A give a marked reaction with the non-specific antigen. They also form relatively less precipitate at 37°C. than do the sera of group B.

The chemical method has the distinct advantage of the elimination of arbitrary standards, since a quantity of antibody protein is directly referable from serum of one type to another.

M12. The Production and Standardization of Diagnostic Antipneumococcal Sera. HAROLD W. LYALL AND HELEN R. ODELL, New York State Department of Health, Albany.

The provision of diagnostic antipneumococcal sera of proven specificity and potency constitutes a highly important problem in connection with the extended use of serum therapy in pneumococcal pneumonias. Experience in the standardization of relatively large amounts of rabbit sera of all the recognized types has afforded considerable data of interest. Cross-reactions occur more frequently in some of the typing sera than in others. Such reactions may be present more or less regularly and may or may not be reciprocal. Other unpredictable cross-reactions also occur. Extensive tests in which representative strains of all types are used are, therefore, necessary before a serum is released for distribution. Sera with cross-reacting properties may be rendered specific by absorption. The practicability of this procedure is, however, yet to be determined. The "quellung" titers of monovalent sera and of mixtures of several types of sera necessary to procure satisfactory results require further consideration before a definite standard can be adopted. The increasing number of strains of pneumococci which cannot be placed in recognized groups, some closely related to established types, presents a problem from the standpoint of the classification of strains and the standardization of sera—also in the evaluation of the results of specific serum treatment. The establishment of an arbitrary number of types to be included in a serum mixture or pool is not essential, provided sera of satisfactory potency and those with reciprocal cross-reactions are combined.

M13. Coccidioidomycosis. Ernest C. Dickson, Stanford University School of Medicine, San Francisco, Calif.

A review of coccidioidal granuloma from a clinical and industrial point of view will be presented. The story of the primary acute infection, which is found in the San Joaquin Valley and which precedes coccidioidal granuloma, will be related. The close resemblance of the primary disease, as well as of the secondary or terminal coccidioidal granuloma, to tuberculosis will be made clear.

The primary infection is caused by the inhalation of the chlamydospores of the fungus. Characteristic pulmonary changes, which are often diagnosed as tuberculosis by roentgen examination, are of relatively short duration. Erythema nodosum is extremely common. The fungus may be recovered from the sputum. The great majority of victims, who think they have had a bad cold or "flu", recover promptly without complications. There is a specific skin-test antigen which indicates unusual hypersensitivity when the patient has erythema nodosum. A small number of the patients, whether they have had erythema nodosum or not, develop the highly disabling and highly fatal coccidioidal granuloma.

Inhalation experiments have shown that the chlamydospores can produce a characteristic pathological reaction in the lungs which differs from the typical granulomatous change of coccidioidal granuloma. Experiments have also explained the formation of the typical spherules and endosporulating forms which are seen in infected tissues.

M14. An Endotoxin from Aspergillus fumigatus. ARTHUR T. HENRICI, University of Minnesota, Minneapolis.

Cell sap from a pathogenic strain of *Aspergillus fumigatus* is toxic for rabbits, guinea pigs, mice and chickens, while the broth filtrate is harmless. Pathogenic strains of *Aspergillus flavus* and *Aspergillus oryzae* show a similar but lesser toxicity. The cell sap is hemolytic *in vitro.* Injected subcutaneously in rabbits, it produces a massive gelatinous exudate similar to that resulting from *Clostridium oedematiens* toxin, with central purpura and necrosis. Intravenously and intraperitoneally, large doses cause death within 48 hours, with pulmonary congestion and hemorrhages; with smaller doses death is delayed up to 2 weeks, and fatty changes with necrosis are found in liver and kidneys. An extensive serofibrinous exudate in pleural or peritoneal cavities is common but not constant. Guinea pigs often develop a paralysis, beginning in the hind quarters and becoming general before death; they are usually found dead in a sitting posture. The toxin is not neutralized by sodium ricinoleate, in fact its action is accelerated. In this, as well as in the nervous symptoms and visceral lesions, the toxin closely resembles that of *Amanita phalloides;* it differs, however, in being heat-labile and non-toxic by mouth. It resists 55°C. for 45 minutes, but is inactivated at 62°C. for 15 minutes. Rabbits and guinea pigs may be immunized actively so that they tolerate 10 or more lethal doses; but a long drawn-out immunization is required, and many animals are lost in the process. The serum of actively immunized rabbits affords passive protection to guinea pigs, and neutralizes the hemolytic action *in vitro.*

M15. On the Spirochetici dal Action of Arsenic and Bismuth Compounds in Vitro. HARRY EAGLE, Johns Hopkins Hospital, Baltimore, Md.

Arsphenamine and some of its derivatives (neoarsphenamine and silver arsphenamine), "arsenoxide", various inorganic arsenicals, and bismuth compounds all immobilize and kill pathogenic *Treponema pallidum in vitro.* Some of these substances have a direct effect on the organism. In the case of others (*e.g.*, neoarsphenamine), the preliminary oxidation by atmospheric oxygen seems to be essential for antispirochetal activity. There are yet other compounds (*e.g.*, sulpharsphenamine) which are almost entirely inert *in vitro,* even in the presence of atmospheric oxygen, which are nevertheless actively therapeutic in human and rabbit syphilis.

M16. Epidemiological Studies on Weil's Disease, with Notes on the Susceptibility of the "Gopher," Citellus richardsonii (Sabine), to the Experimental Inoculation of Leptospira icterohemorrhagiae.

JEROME T. SYVERTON, W. W. STILES AND GEORGE PACKER BERRY, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It has been found that the "gopher" or Richardson ground squirrel, *Citellus richardsonii* (Sabine), is susceptible to the etiological agent of Weil's Disease, *Leptospira icterohemorrhagiae.*

A human and a rat strain of this organism were used to initiate in gophers 2 transmission series which were carried through 15 and 5 successive passages, respectively. Transfers of the organism were effected by the parenteral introduction of blood or kidney-suspension derived from the preceding host. Following the final passage, the organisms were recovered and their unaltered pathogenicity for guinea pigs was demonstrated. Transfers of the infection were also accomplished by permitting normal gophers to feed on their cage mates, which were moribund or dead of experimental Weil's Disease. It is suggested, therefore, that the gopher and similar wild rodents may act in nature as intermediate hosts for the spirochete.

With the aid of this highly susceptible new host, and of a previously described simple laboratory procedure which uses guinea pigs for the detection of strains of *Leptospira icterohemorrhagiae* of low virulence, we have isolated 27 strains of this organism from 170 wild rats and 2 strains from human cases of Weil's Disease. The rats were obtained in Rochester, N. Y., (18 of 47 rats positive), Detroit, Mich., (7 of 42 rats positive), and San Francisco, Calif., (2 of 12 rats positive). Neither human cases of Weil's Disease nor the carrier state in rats has been established previously in Detroit or Rochester.

M17. The Inactivation of Gonococcal "Toxin" in Vitro by Sulfanilamide.

C. M. Carpenter, G. M. Barbour and P. L. Hawley, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Recently we have observed that sulfanilamide will protect mice against lethal amounts of "toxin" prepared from a strain of *Neisseria gonorrhoeae* isolated from the knee joint of a patient with gonococcal arthritis. Because of this finding *in vivo*, tests were made to determine whether sulfanilamide had an effect on gonococcal "toxin" *in vitro*. The "toxin" employed was simply a lyophilized and regenerated whole 6-day ascitic fluid broth culture of the above strain of gonococcus. Since we had found that 10 mg. of sulfanilamide were required to protect mice against 0.1 cc. of this material, mixtures of sulfanilamide and "toxin" were prepared in this ratio. The mixtures were used after incubation at 37°C. for 24 hours. Their pH values did not change significantly during incubation. Mice in groups of from 15 to 50 were injected with the sulfanilamide-"toxin" mixtures. Controls were provided by mice in similar groups which received untreated "toxin," *i.e.*, "toxin" unmixed with sulfanilamide, but incubated at 37°C. for 24 hours. Combining the mortalities noted in the various tests, it was found that only 25 per cent of the 295 mice injected with the sulfanilamide-"toxin" mixtures died, while all of the 116 control mice injected with the untreated "toxin" succumbed.

M18. The Cultivation of Neisseria gonorrhoeae on the Chorio-Allantoic Membrane of the Chick Embryo and the Use of This Technic for the Study of Sulfanilamide.

Grant Morrow and George Packer Berry, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The lack of any animal which is susceptible to infection with *Neisseria gonorrhoeae* has greatly hindered the study of gonococcal infections. Since several types of highly parasitic bacteria have been cultivated on the chorio-allantoic membrane of the chick embryo, it seemed important to ascertain whether this *in vivo* technic would succeed in the case of the gonococcus. Such proved to be so. Using the method of Goodpasture and Buddingh, we have been able to grow both old laboratory and freshly isolated strains of *Neisseria gonorrhoeae*. Each of 2 old and 2 new strains has been readily carried through 25 and 10 egg-transfers, respectively. The characteristics of the organisms have not changed perceptibly. In the eggs, the infection seems to be localized to the chorio-allantoic membrane; apparently the embryo has not been invaded.

The successful cultivation of gonococci on the chorio-allantoic membrane has enabled us to study *in vivo* the prophylactic and therapeutic effects of sulfanilamide. When 0.3 cc. of a 1.6 per cent solution of the drug were dropped on the chorio-allantoic membrane 24 hours before inoculation with gonococci, no infection occurred. When the drug was used therapeutically after a gonococcal infection had been established for 24 hours, however, the amount of sulfanilamide required to eradicate the organisms was twice the prophylactic dose. These statements are based on a number of experiments with 2 old laboratory strains. It is obvious that this method provides an opportunity for testing other chemotherapeutic agents.

M19. Experimental Staphylococcemia in White Mice Produced by the Intraperitoneal Injection of Mucin-Suspended Staphylococci.

B. S. Levine, National Institute of Health, U. S. Public Health Service, Washington, D. C.

Preliminary to the initiation of studies in chemotherapeusis of staphylococcemias, attempts were made to develop a method for the production under controlled conditions of experimental staphylococcemia in white mice.

Series of dilutions of 5-hour staphylococcal broth cultures were made in saline alone, for control purposes, and in mucin, as described by Miller. Doses of 0.5 cc. of each were injected intraperitoneally into white mice. Mucin increased the susceptibility of the mice, but not to as great a degree as was expected. When the dosage and the animal's susceptibility were favorable, death occurred within 48 hours and, in the absence of contaminant-invasion, was due primarily to the effect of the exostaphylotoxin and secondarily to the staphylococcal invasion. Mice dead as a direct or indirect result of the experimental inoculation were autopsied as soon as possible. Mice surviving for one week were etherized and autopsied at once. Bacteriological studies were made of the heart-blood of all mice.

The rapid death of mice injected with high dilutions of the staphylococcal broth culture in mucin, or death occurring several days after injection with low dilutions, in nearly every instance, was due to the accidental presence of enteric bacteria, either in pure culture or in dominant admixture. Contamination with bacteria culturally simulating *Eberthella enterica*, *Salmonella icteroides*, and *Salmonella abortivo-equina* proved most fatal to the mucin-injected white mice.

In the surviving mice, the immunological mechanism appeared to be two-fold: (1) serum immunity, with heart-blood sterile, and (2) localization in pure culture of the original staphylococci in abscesses.

M20. Failure of Vitamin C to Potentiate Formed Antigens. R. R. MADISON AND W. H. MANWARING, Stanford University, Calif.

We have previously shown that the injection of horse serum plus ascorbic acid (or in association with this enzymic activator) gives a ten-fold greater yield of antihorse precipitins in rabbits than that obtained after control injections with non-potentiated horse proteins. Vitamin C, however, does not increase the yield of antibodies against formed elements. Ninety-six rabbits have been injected in groups with such formed antigens as sheep erythrocytes, *Eberthella typhosa*, *Proteus vulgaris*, or *Diplococcus pneumoniae*. Without exception, agglutinin, precipitin and Forssman-antibody formation were identical in the Vitamin C-potentiated rabbits and in the control animals. This observation suggests the tentative conclusion that two different mechanisms for the specific formation of antibodies must be operative in rabbits; one capable of potentiation (or stimulation) by Vitamin C, the other refractory to this enzymic activator.

M21. An Outbreak of Botulism in Captive Mink on a Fur Farm in Colorado. IVAN C. HALL AND GEORGE W. STILES, University of Colorado School of Medicine and Hospitals and U. S. Bureau of Animal Industry, Denver.

The death of 146 out of 148 mink on a fur farm near Denver, Colorado, was shown to have been caused by the toxin of *Bacillus botulinus* (Type A). This is believed to be the first recorded instance of naturally occurring botulism in mink or other fur-bearing animals. The financial loss, after salvage of the pelts, was approximately $7,000. The toxin was demonstrated in filtrates of scrapings from the feeding boards, of the stomachs of the dead mink, and of their feces. It proved to be impossible to trace the origin of the toxin. All of the examinations of suspected foods, frozen hog livers, commercially canned fish of two brands and commercially canned tomato purée gave negative results for toxin.

M22. Atypical Gram-Negative Organisms from Cases of Acute Intestinal Disorders. F. E. Colien, Creighton University School of Medicine, Omaha, Neb.

Stool specimens from cases of acute intestinal disturbance and autopsy material from one case were examined on modified Endo's and eosin-methylene blue media and on Entogenic Agar (Bacto). Gram-negative short rods more nearly resembling members of the dysentery group than any other, yet differing from any of the recognized members of that group in some biochemical or serological reaction, were isolated from all cases, in some instances in pure culture. The biochemical reactions of these organisms were studied in relation to strains of *Shigella paradysenteriae* (Flexner variety), *Shigella paradysenteriae,* var. *sonnei, Shigella dispar, Shigella ambigua* (Schmitz bacillus) and *Shigella alkalescens.* The antigenic relationship of these organisms to the strains of dysentery bacilli and to members of the coli group was also investigated. The blood sera from several of the cases agglutinated the organisms described in a dilution of 1:80.

M23. A Study of Strains of Shigella Isolated from Urine. Luther Thompson, Mayo Clinic, Rochester, Minn.

In a series of 5,000 cultures of urine which were made in 1935, about 0.5 per cent of the cultures contained organisms belonging to the genus *Shigella.* During the period between September, 1937, and March, 1938, 13 strains of *Shigella* were isolated from urine and studied by cultural and serological methods. All belonged to the mannite-fermenting group and were negative in lactose and sucrose. Most strains fermented dulcitol and xylose and produced indol. All strains were non-motile and failed to liquefy gelatin.

Agglutination tests with sera prepared by immunizing rabbits with 4 separate strains showed that the cultures were antigenically heterogeneous. Unless some contrary evidence can be found, it would seem best to consider these organisms as strains of *Shigella alkalescens* showing minor variations in fermentation. Agglutination tests indicate that there are 2 serological groups which include 7 of the 13 strains.

M24. Viruses and Virus Diseases. Thomas M. Rivers, Hospital of the Rockefeller Institute for Medical Research, New York City.

For a long time many workers refused to admit that virus diseases should be placed in a special class and that the viruses themselves should be looked upon, except for size, as a special group of infectious agents. At present most workers agree that there is a special group of agents different from ordinary protozoa, fungi, bacteria, spirochetes and rickettsiae. However, there is no general agreement regarding the nature of the viruses.

The outstanding work of Stanley, in which a nucleoprotein composed of extremely large molecules and possessed of all the characteristics of the causative agent of tobacco mosaic was obtained in crystalline form from the sap of diseased plants, has struck the imagination of a host of workers. Many of these investigators have rushed to the conclusion that all viruses are macromolecules.

Stanley's findings have been abundantly confirmed, but the origin of the macromolecules and the mode of their reproduction are not known. Some of the explanations are fanciful and one should be slow to accept them without more convincing evidence. Furthermore, there is already sufficient evidence that some of the viruses, *e.g.*, those represented by elementary bodies, are entities of a much greater complexity than are Stanley's macromolecules. Consequently, it is not wise at present to make too many generalizations regarding the nature or mode of reproduction of the agents placed in the virus group, because no evidence has been brought to show that all of them must be identical or similar in nature.

M25. Antigenic Differences in Strains of Epidemic Influenza Virus.

Thomas Francis, Jr., and T. P. Magill, International Health Division Laboratories, Rockefeller Foundation, New York City.

In 1937, Magill and Francis reported that 2 strains of epidemic influenza virus which had appeared to be identical immunologically could be differentiated serologically. These studies have been extended to embrace observations on the antigenic constitution of 24 strains of the virus. Two methods have been employed: (1) cross-neutralization tests using the various strains of virus and sera prepared against these strains by the intraperitoneal vaccination of rabbits; (2) cross-active immunity tests in mice immunized by the intraperitoneal inoculation of virus. By the use of these procedures it has been possible to show that distinct differences in strains of epidemic influenza virus are detectable. In some instances the serological differences are quite marked; in other instances, extremely slight. The significance of these variations will be discussed.

M26. A Soluble Antigen from Infectious Myxomatosis in Rabbits.

THOMAS M. RIVERS, S. M. WARD AND J. E. SMADEL, Hospital of the Rockefeller Institute for Medical Research, New York City.

The occurrence of a soluble antigen in virus-free filtrates prepared from infected skin and from serum of rabbits sick with myxoma has been previously reported. Results of present studies on the nature of this antigen are summarized in the following manner: it is destroyed by a temperature of 56°C., by hydrogen ion concentrations greater than pH 4.0, or by formaldehyde in concentrations greater than 0.5 per cent; it is a globulin with an isoelectric point close to pH 4.5; it is not appreciably affected by digestion with commercial trypsin; on storage gradual loss of ability to form a precipitate with myxoma-immune serum occurs. Purification of the antigen was attained by the precipitation of the crude preparation at different pH values, with or without ammonium sulfate, followed by tryptic digestion.

Partially purified antigen, which was obtained from serum and which reacted with immune serum in a dilution of 1:10,000, gave no evidence of the presence of nucleic acid, gave a faintly positive Molisch reaction, contained 15.4 per cent of nitrogen, and on spectroscopic examination showed bands typical of the aromatic amino acids present in ordinary globulins. Rabbits immunized with partially purified antigen developed in their serum precipitins against the antigen, but responded as did normal animals to inoculation with the viruses of myxoma, neuromyxoma or fibroma.

M27. A Non-Lethal "Mutant" Strain of Virus myxomatosum Derived from Fibroma Virus.

GEORGE PACKER BERRY, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

A non-lethal "mutant" strain of *Virus myxomatosum,* named "80-A", has been obtained by transformation from the virus of rabbit fibroma (Shope). This "80-A" strain came from a rabbit inoculated with a mixture of active fibroma virus (Cayuga strain) and heat-inactivated myxoma virus (Brazilian). The fibroma virus was employed as a 1 per cent suspension of fibromatous testicle, the inactivated myxoma virus as a suspension of washed elementary bodies (titer 10^7) which had been heated at 80°C. for 30 min.—a procedure which is invariably lethal for myxoma virus and which usually inactivates the "transforming agent".

The word "mutant" is used in describing the "80-A" virus, because the strain appeared suddenly with distinctive characteristics which have persisted for 13 months through 23 passages in rabbits. The most striking feature of "80-A" myxomatosis is the low mortality rate (15 per cent from the uncomplicated infection). In the rabbit, both clinically and pathologically, the condition is easily differentiated from fibroma or ordinary myxomatosis. It also has distinguishing characteristics on the chorio-allantoic membrane of the chick embryo. Among other differences, it has been more difficult to cultivate than myxoma virus. After a few passages in eggs, it has behaved typically on retesting in rabbits. A "transformation experiment" using a heat-inactivated suspension of the elementary bodies of "80-A" virus (titer 10^7; 65°C. for 30 min.) and active fibroma virus (Cayuga) has yielded a modified "myxoma" virus which is apparently identical with "80-A". Its characteristics have persisted for 4 months through 7 passages in rabbits.

M28. A Further Study of the Cultivation of Virus myxomatosum on the Chorio-Allantoic Membrane of the Chick Embryo. RACHEL E. HOFFSTADT AND K. STEPHEN PILCHER, University of Washington, Seattle.

Recently Hoffstadt and Pilcher reported the cultivation of *Virus myxomatosum* on the chorio-allantoic membrane of the chick embryo. Further studies have been made on the factors influencing the growth and reaction of the virus in the embryo. The temperature for optimal development was found to be from 33° to 35°C. The virus, after many serial passages, apparently adapted itself to growth within the egg. The virus produced infection in the embryos, as indicated by gross pathological changes, changes in the blood counts and in a reduction in the percentage of hatch of the eggs.

M29. Effect of Solutions of Sucrose, Glycerol, and Urea on the Size and Density of the Elementary Bodies of Vaccinia. J. E. SMADEL, E. C. PICKELS AND T. SHEDLOVSKY, Rockefeller Institute for Medical Research and International Health Division Laboratories, Rockefeller Foundation, New York City.

The rate of sedimentation of the elementary bodies of vaccinia was studied in the ultra-centrifuge of Bauer and Pickels with the light-absorption method of Svedberg. In dilute buffer solutions with pH values close to 7.0, the single virus particles sediment at a rate of approximately 49×10^{-11} cm./sec./dyne. The rate of sedimentation of the elementary bodies increases after suspension in solutions of sucrose, glycerol, and urea. Different maximal rates are obtained in these solutions having the same specific gravity. The maximal rate is generally maintained in solutions of sucrose, but in concentrated urea it subsequently declines. The sedimentation rate returns to normal when the elementary bodies are resuspended in dilute buffer solution.

The density of elementary bodies suspended in dilute buffer is estimated as 1.16 gm./cc. However, the density is increased by contact with solutions of sucrose, glycerol, or urea. For example, it becomes 1.25 gm./cc. in a 53 per cent solution of sucrose. With sucrose, the increase in density may be accounted for by an osmotic extraction of water from the particles. On this basis one-third of the volume of the particle is extracted by a solution of sucrose of specific gravity 1.25. The behavior of the elementary body in solutions of urea is complicated by its permeability to urea. The effect of glycerol is intermediate between that of sucrose and urea.

M30. Experiments on Antirabic Immunization. ANSON HOYT, FREDERICK J. MOORE, M. KATHERINE GURLEY AND DOUGLAS WARNER, University of Southern California School of Medicine, Los Angeles.

The sera of goats and rabbits, hyperimmunized with rabies fixed virus, were tested for neutralizing and protective properties. White mice were employed as the test animals, all virus inoculations being given by the intracerebral route. Both goat and rabbit antisera possessed high neutralizing titers against fixed virus. In experiments on protection, the rabbit serum proved greatly superior to goat serum. Rabbit serum injected intraperitoneally before the inoculation of street virus brought about an extremely high percentage of survival, whereas almost all untreated control animals contracted rabies. Rabbit serum also demonstrated considerable protective effects even when administered as late as four days after street virus.

Experiments on combined passive and active immunization against fixed virus showed that a single injection of antirabic rabbit serum, given at the beginning of active immunization, largely nullified the effects of 12 daily doses of vaccine. Mice receiving vaccine alone began to demonstrate active immunity immediately following 5 daily injections. Further vaccination produced an immunity of marked degree. Mice could not be protected against fixed virus, if active immunization were begun after the inoculation of virus. However, it was possible to protect mice when vaccination was started after the administration of street virus. A possible explanation lies in the fact that fixed virus begins to multiply rapidly in the brains of mice about two days after inoculation, whereas it takes approximately six days for the demonstrable multiplication of street virus.

M31. Plant Viruses as Factors in Morphogenesis. MICHAEL SHAPOVALOV, Bureau of Plant Industry, U. S. Department of Agriculture, Logan, Utah.

Viruses tend to alter host-plant morphology normally controlled by genes and environment. Many of these virus-controlled changes can be transmitted by insects, grafting, or the injection of juice.

The morphogenetic effects of viruses may be largely referred to the sphere of teratology. In their manifestation these effects are either suppressive, as in dwarfing or the filiformity of normally broad-leaf blades, or stimulative, as in excessive vegetative growth, protuberance formation or leaf branching. Infectious leaf variegation is a form of chlorophyl-suppressing activity of the virus. When the suppressing effect of a virus is partially overcome by normal processes of the plant, peculiar manifestations of suppressed recovery result, as in recovery from curly top virus.

Virus-induced malformations often parallel aberrant genetic forms. Thus, "shoestring" tomato mosaic closely resembles "wiry" mutant. Leaf variegation may be caused by either virus or genetic factors, and leaf branching may arise from either genetic or virus causes.

As teratological phenomena, readily obtainable and easily controlled, virus-induced malformations may be of great value in experimental morphology. In some cases they present the best means for revealing the plant's phylogenetic history.

The application of form-building viruses in ornamental horticulture, although rare and limited to certain specific forms, is nevertheless established and has produced such plants as some of the variegated abutilons, tulip Rembrandt, etc. Further possibilities in this respect, particularly with reference to the culture of dwarfed plants, deserves attention.

M32. Studies on Selected Strains of Curly Top Virus. N. J. GIDDINGS, Bureau of Plant Industry, U. S. Department of Agriculture, Riverside, Calif.

Four strains of curly top virus, designated 1, 2, 3, and 4, were recognized by the differential reactions of varieties of sugar beet (*Beta vulgaris* L.). Percentages of plants infected and severity of symptoms were used as bases of comparison. Strains 1 and 3 induced severe symptoms in susceptible beets, but strain 3 seldom infected the resistant beet used. On the other hand, strain 1 infected a high percentage of resistant beets and induced obvious symptoms. Strains 2 and 4 induced mild symptoms in susceptible beets, but strain 4 infected only a small percentage of the resistant beets. On the contrary, strain 2 infected a high percentage of the resistant beets, but induced mild symptoms. The highly-resistant beet variety, known as 1167, showed distinctive reactions to each of the 4 strains of virus and the differences were highly significant statistically. Tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* Mill.) were not infected by strains 2 or 4. Plantain (*Plantago erecta* Morris), peppergrass (*Lepidium nitidum* Nutt.) and Great Northern variety of bean (*Phaseolus vulgaris* L.) were not infected by strain 2. Susceptible beets infected with the less virulent strains were not immunized against the more virulent strains, but appeared rather to be rendered more susceptible to severe injury by such prior infection.

M33. Control of Plant Virus Diseases in California. Henry H. P. Severin, University of California, Berkeley.

Several methods are available for controlling curly top of the sugar beet and the beet leafhopper, *Eutettix tenellus* (Baker). One depends on the use of a definite planting schedule for sugar beets. This schedule, designed to avoid curly top in natural breeding areas, is related to the spring and autumn dispersal of the insects. Another method employs a variety of sugar beet, developed by the United States Department of Agriculture, which is resistant to curly top. Other lines of attack designed to control the beet leafhopper are (1) control by spraying (based upon the fact that the leafhoppers are forced to congregate in large numbers upon vegetation in dry washes during the autumn), (2) control by destroying the principal summer host plant, the Russian thistle (*Salsola kali-tenuifolia*), by summer hoeing, dragging, or disking, and (3) control through the ultimate elimination of the Russian thistle by preventing its spread and reducing its seed supply by burning the mature thistle stands late in the autumn before the plants are torn loose and start rolling.

A practical method for combating western celery mosaic involves adopting a celery-free period in the fields varying from 3⅔ to 5 months and in the greenhouses from 1 to 1⅔ months. All fields in the restricted area are plowed and all plants not previously harvested are destroyed. No celery is allowed to be shipped into these districts from other localities.

In Colorado it has been demonstrated that peach mosaic may be controlled by the removal of diseased trees. Similar methods are now being used in California.

M34. Detection of the Virus of Poliomyelitis in the Nose and Throat and Gastrointestinal Tract of Humans and Monkeys. SIDNEY DAVID KRAMER, Long Island College of Medicine, Brooklyn, N. Y.

Nasal washings and stools from 20 patients convalescent from poliomyelitis, and nasal washings, scrapings and intestinal contents from 7 monkeys sacrificed at the height of the experimental disease, were studied. These materials were treated with ether and concentrated *in vacuo*. The concentrated materials were inoculated intracerebrally and intraperitoneally into normal monkeys. Animals presenting suggestive symptoms were sacrificed and serial passages to second animals were made. Positive detections of the virus were conceded when the monkeys presented symptoms and histologic findings compatible with experimental poliomyelitis.

The virus was detected in 4 instances from human sources and in 1 instance from a monkey. Of the 4 human strains, the first was obtained from the feces of a child 7 days after onset of illness. The second strain was obtained from the nasal washings of a child 5 days after onset. The third and fourth human strains came from the same patient 9 days after onset, from the nasopharynx and the feces, respectively. The single strain recovered from a monkey was obtained from the gastrointestinal tract.

In view of the normal swallowing of oral and nasal secretions, it is suggested that recovery of the virus from the human gastrointestinal tract need not alter the concept that the virus normally enters the body by way of the upper respiratory tract.

M35. Influence of Sterile Inflammation on the Nasal Portal of Entry in Poliomyelitis. P. F. CLARK, K. E. LEMMER AND A. F. RASMUSSEN, University of Wisconsin Medical School, Madison.

It is now commonly admitted, on the basis of much evidence, that the usual portal of entry in poliomyelitis is through the olfactory mucosa.

Lennette and Hudson found that sectioning of the olfactory tracts of monkeys prevented the disease, even when the intravenous route, with large doses of virus, was employed. Similarly, Armstrong protected animals against intravenous injections by treating the nasal mucosa with picric acid.

In a series of 8 *Macacus rhesus* monkeys, the olfactory tracts of 4 were sectioned, while 4 were left normal. After several weeks had elapsed for recovery, a sterile inflammation was produced in 2 of the sectioned animals and in 2 of the normal controls, by injecting 1 cc. of 1 per cent starch paste into each frontal lobe, and 2 cc. of normal horse serum intraspinally by lumbar puncture. On each of the next 3 days, each of the 8 animals were injected intravenously with 10 cc. of supernate from a 5 per cent centrifuged suspension of glycerinated cord (30 cc. total dose). Only the 4 monkeys with the sterile inflammation developed poliomyelitis.

It seems probable that this inflammation caused sufficient injury to the blood central-nervous-system barrier to permit the virus to pass directly to the brain and cord. It is clear, therefore, that an otherwise subinfective intravenous dose was rendered infective by a sterile inflammation, whether the olfactory tracts were sectioned or not. The intact olfactory tract was not, apparently, a determining factor.

M36. *Relation of the Concentration of Virus to the Pathogenesis of Poliomyelitis.* E. W. SCHULTZ AND L. P. GEBHARDT, Stanford University, Calif.

Recent investigations on the kinetics of bacteriophagy indicate that bacteriophage may be formed by intact living bacteria and that lysis may not occur until a certain "critical" concentration of the lytic principle has been reached. We have made observations which suggest that in poliomyelitis there is a similar relationship. Titration studies during the preparalytic stage of the experimental disease show that the virus may reach high concentrations in certain portions of the nervous system without producing conspicuous nerve-cell damage and may attain a high concentration in the cord before paralysis is manifested. In fact, its concentration in the cord at the time of paralysis may be lower than that reached in the olfactory bulbs and hypothalamus, in which less nerve-cell damage seems to occur.

Briefly, our observations suggest that the concentration of virus is not necessarily directly related to nerve-cell destruction and that virus can be formed in large amounts in certain areas without producing extensive nerve lesions; also that paralysis is due not so much to a particular mobilization of the virus in the cord and medulla as to an inherently greater susceptibility of anterior horn-cells.

It seems probable that, like strains of bacteriophage which despite serial passage remain of low titer and low lytic power, there are "low titer" strains of poliomyelitis virus, which although not lacking in invasiveness may be unable to build up a concentration of virus high enough to damage nerve-cells. Naturally acquired immunity may be based largely on exposure to such strains.

M37. *Variations in Strains of Poliomyelitis Virus.* Fred D. Stimpert and John F. Kessel, University of Southern California School of Medicine and Los Angeles County Hospital, Los Angeles.

Five strains of poliomyelitis virus isolated in Los Angeles have been compared with two Eastern strains, the M.V. strain and one isolated recently by Dr. L. W. Aycock in Boston. The comparison was made by means of cross-neutralization tests using sera of monkeys recovered from infection. A minimum of 10 animals was used in an experiment with each virus. The final suspension of virus in each injected dose of serum-virus mixture, as determined by the titration of pools, contained 10 minimal lethal doses. The dilution of serum used was 1-10.

Wide ranges of virulence were noted in animals infected with different strains, such differences being independent of the immunologic differences shown by neutralization tests. It is indicated that the development of neutralizing antibodies against homologous virus in the serum of monkeys recovering from infection was quite regular, and that the sera of animals recovering from the less severe disease demonstrated, in general, greater neutralizing power.

A closer relationship is noted between strains from the Los Angeles area than between these and the strains from other areas. Immunologic differences, however, do occur within the group of recently isolated strains from Los Angeles. Differences are also apparent between recently isolated strains and the M.V. strain, and between the M.V. strain and the strain from Boston. Neutralization may occur in one direction without reciprocation.

In summary, it would appear that these indications of immunologic variations are due to differences in antigenic structure rather than to the fact that a certain strain is a recently isolated one, an old passage strain, or one related to a particular epidemic.

M38. Neutralization Test with the Sera of Poliomyelitis Patients from the Recent Epidemic in Los Angeles. JOHN F. KESSEL, FRED D. STIMPERT AND ROY T. FISK, University of Southern California School of Medicine and Los Angeles County Hospital, Los Angeles.

Sera collected from poliomyelitis patients in the Los Angeles County Hospital during the period from 1934 to 1936 were employed in neutralization tests with the M.V. strain of virus from the Rockefeller Institute, the McK strain isolated in Los Angeles in 1935, the St. strain isolated in Los Angeles in 1936 and the Bo strain isolated by Aycock in Boston in 1936.

Of 41 onset and convalescent sera compared, 28 per cent of the onset sera and 50 per cent of the convalescent sera neutralized M.V. virus, while 48 per cent of the onset sera and 84 per cent of the convalescent sera neutralized the local McK virus.

In comparing the convalescent sera from 37 children and from 35 adults, it was found that 38 per cent of the children and 70 per cent of the adults neutralized the M.V.virus, while 70 per cent of the children and 97 per cent of the adults neutralized the local McK virus.

In comparing the convalescent sera of 38 patients with mild symptoms and 29 with severe symptoms, it was found that 53 per cent with mild symptoms and 55 per cent with severe symptoms neutralized M.V. virus, while 72 per cent with mild symptoms and 92 per cent with severe symptoms neutralized the McK virus.

In comparing the convalescent sera with the 4 strains of virus, it was found that 40 per cent neutralized the M.V. strain, 50 per cent neutralized the St. strain, 30 per cent neutralized the Bo strain, and 90 per cent neutralized the McK strain. Variations of neutralization results, using the same sera but different strains of virus, are thus noted.

M39. Immunological Reactions in Poliomyelitis. SIDNEY RAFFEL, Stanford University, Calif.

Studies were made in an attempt to demonstrate reactivity between poliomyelitis immune sera and the virus or its products in nervous tissue. These investigations were directed toward a further analysis of the immunologic properties of the virus, as well as toward simplification of the identification of infection or the recognition of acquired immunity. The procedures involved the testing of virus and normal cord suspensions, extracts and fractions, variously concentrated, by active and passive sensitivity tests in guinea pigs and monkeys; also by precipitation and complement-fixation tests and by Shwartzman reactions in rabbits.

With most of the methods employed, there was no indication of an immunologic distinction between infected and normal cord preparations. Specific reactivity has been noted, however, in a limited number of sensitivity tests carried out with ultracentrifuged cord sediments. Since such sediments have previously been shown to contain relatively high concentrations of the virus, further confirmatory work is being carried on. It is planned, also, to determine whether the virus itself is responsible, or whether some other cord element takes part in the reaction.

M40. Neutralization and Complement-Fixation Tests with Four Neurotropic Viruses and Human Sera Collected in California during 1937 and 1938. BEATRICE HOWITT, Hooper Foundation for Medical Research, University of California, San Francisco.

Sera were collected in California from regions where cases were reported as having recovered from either a typical encephalitis or a "polio-encephalitis" during the summer of 1937. A few had been diagnosed during 1936.

The neutralization test showed that 40.3 per cent of 57 sera were positive against St. Louis encephalitis virus. All but one of the positive cases resided in some portion of the two large central California valleys, the majority around Fresno and Tulare (30 cases with 56.6 per cent positive). The same sera were also tested against the viruses of poliomyelitis, of lymphocytic choriomeningitis and of Japanese B encephalitis. Tests with the two latter were negative, but 16 (51.6 per cent) of 31 sera neutralized the virus of poliomyelitis

About 76 of the sera from a group of cases diagnosed as having had poliomyelitis without residual paralysis in Tulare County since 1934 gave positive neutralization against poliomyelitis virus. A certain number of them were also positive against St. Louis encephalitis virus, thus indicating the presence of this disease for a longer period than had been suspected. No positive tests for this latter virus were obtained with sera from individuals who had had typical poliomyelitis with residual paralysis. Normal sera, with the exception of that of a nurse from Fresno, were negative.

It was found feasible to use the complement-fixation test as a supplementary aid in the diagnosis of St. Louis encephalitis. Many of the sera showed definite correlation between positive neutralization and positive complement fixation. All normal sera and those from paralytic cases of poliomyelitis, however, have been negative.

M41. Production of Antiserum for Equine Encephalomyelitis. EDWARD RECORDS AND L. R. VAWTER, University of Nevada, Reno.

Antisera of high virus-neutralizing titer will probably be needed for some time to come as an aid in the treatment of clinical cases of equine encephalomyelitis. Even after a prolonged course of immunization by the subcutaneous injection of virus into horses, the response is poor or variable and the sera of only a few animals attain a titer considered satisfactory for clinical use. We have recently observed that a single intravenous injection of a large dose of equine encephalomyelitis virus into immune horses results in a ten-fold rise of antiviral titer in 10 days. So far, we have encountered no fatalities or unduly severe reactions in the injected horses following this procedure. Titration of the virus-neutralizing power of individual and pooled sera from hyperimmune horses has been conducted on both 10-day chick embryos and guinea pigs. The chick embryo is considered equally, if not more, dependable than guinea pigs. The use of the embryos, moreover, is decidedly more economical, and the procedure is more rapid.

M42. Heterogeneity of Coliform Bacteria as Shown by the Absorption of Bacteriophage. PHILIP L. CARPENTER, Iowa State College, Ames.

On the basis of recent work, it may be assumed that the specificity of the absorption of bacteriophage is dependent upon the factors that determine serological specificity. Therefore, an attempt was made to separate 127 strains of coliform organisms into groups based on the absorption of anti-coli and anti- "Citrobacter" bacteriophages. The coliform strains were representative of *Escherichia*, *Aerobacter*, "Citrobacter" and Voges-Proskauer-positive "intermediates". The 4 groups separated by absorption did not correspond even closely to the grouping of the strains on a physiological basis. There were indications of marked serological heterogeneity.

M43. Specificity of Bacteriophage for Strains of the Escherichia-Aerobacter Group and for Certain Other Members of the Bacteriaceae. M. J. POWERS, MAX LEVINE AND C. S. MCCLESKEY, Iowa State College, Ames.

Bacteriophages were isolated from sewage and packing-house waste against 11 strains of *Escherichia;* 8, *Aerobacter;* 8, *Escherichia-Aerobacter* intermediates; 2, *Proteus;* 1, *Salmonella;* 2, *Eberthella;* 1, *Alkaligenes;* 1, *Klebsiella;* 2, *Flavobacterium;* 2, *Serratia;* and 1, *Achromobacter.* The bacteriophages were purified by repeated "picking" from isolated plaques on agar in Petri dishes. Tests for lysis were made by smearing a young bacterial culture over the surface of agar in a Petri dish, and then streaking the bacteriophage to be tested across the surface of this inoculated substrate. Each bacteriophage was tested against the strains listed above, also against a considerable number of additional cultures of the *Escherichia-Aerobacter* group and other bacteria.

Bacteriophages isolated against strains of the genus *Escherichia* showed a marked tendency to lyse organisms in that genus, but not strains of the genus *Aerobacter,* and *vice versa.* Bacteriophages for *Escherichia-Aerobacter* intermediates showed a moderate tendency to specificity for that group of cultures. Rather specific bacteriophages appear to exist for bacteria in each of the genera *Proteus, Flavobacterium, Serratia* and *Klebsiella.* Some bacteriophages attacked only *Escherichia-Aerobacter* strains, while others attacked both these strains and certain intestinal pathogens as well.

M44. Observations on a Mixed Strain of Staphylococcal Bacteriophage.

EUGENE MAIER, Florida Medical Center, Venice.

A mixed *Staphylococcus aureus* bacteriophage has been employed for about a year for the production of "specific" bacteriophage for use in the treatment of cases of osteomyelitis. In a recent case originating in the calcaneus, all attempts to obtain a "specific" bacteriophage were made in vain. The bacteriophage failed to reproduce, so that none was demonstrable after a few passages. At this point, the widely known and used *Staphylococcus aureus* (205), Department of Agriculture, Washington, was found to be readily lysable. This staphylococcal strain has been kept on agar for a period of 10 years. An attempt was made to obtain lysis from the osteomyelitic strain by pooling stock bacteriophage and the bacteriophage recovered from *Staphylococcus aureus* (205). Complete lysis occurred in the second transfer, the tubes of broth remaining clear for 12 hours. The addition to the stock bacteriophage of dimethyl-benzyl-ammonium chloride in a concentration of 1:100,000 apparently had no deleterious effect on the lytic principle.

M45. Further Studies on the Development of Immunity by Various Tissues.

LORE MARX AND REUBEN L. KAHN, University of Michigan Hospital, Ann Arbor.

Rabbits were immunized by an intravenous injection of horse serum and, after different intervals, various tissues of the rabbits were tested for their capacity to retain injected antigen. These tests were made by the simultaneous injection of horse antitoxin into the tissue to be tested and of a standard dose of homologous toxin subcutaneously. The excess of antitoxin necessary to protect a horse serum-immune animal as compared with the protective dose for a normal animal was the measure of the amount of horse serum protein retained in the tissue. Highest retaining capacity was found in one or another tissue, depending upon the immunizing dose and the interval following immunization.

After immunization with 0.001 cc. of horse serum, the skin and peritoneum developed a high capacity to retain horse antitoxin. The response of the peritoneum was more rapid and marked, but subsided sooner than the cutaneous response. Skeletal muscle retained but very little antitoxin. If 1 cc. of horse serum were used for the immunizing injection, the retention of antigen in all three tissues was higher and lasted longer, but appeared more slowly, and the skin showed a higher retaining capacity than the peritoneum.

M46. A Study of Natural and Acquired Immunity to Staphylococcal Toxin in Monkeys. CHARLES WEISS AND J. D. TARANIK, Mount Zion Hospital, San Francisco, Calif.

The staphylococcal anti-α-hemolysin of the serum of 56 normal monkeys (*Macacus irus* and *Macacus mulatta*) varied from 0 to 25 international units. Intraperitoneal injection of from 10,000 to 20,000 units of Squibb's staphylococcal antitoxin (concentrated horse serum) raised these titers within 48 hours after administration to from 80 to 150 units, the average being about 100. Antitoxin was eliminated rapidly from the blood stream of the monkeys. Thus, there were precipitous drops in titers from 24 to 48 hours after they reached a peak. After a month, the titers had returned approximately to their original levels.

Intradermal or intrabronchial immunization with toxin protected monkeys against several minimal lethal doses of this antigen, when administered intrabronchially, and caused a marked reduction in the severity of cutaneous reactions to test doses. There was much variation in the capacity of animals to develop antihemolysin. The titers reached varied directly with the original (natural) levels.

As in the Schick reaction with diphtheria toxin, there was no fixed antitoxin titer (anti-α-hemolysin) above which animals showed an absence of dermonecrosis, and below which they were positive in this respect. Nevertheless, there was a much higher level of circulating antihemolysin (10 to 60 units) in groups of negative than in groups of positive monkeys, both in their normal state and after active or passive immunization.

M47. Studies on Anaphylaxis in the White Mouse. Russell S. Weiser, Orville Golub and Dorothy Hamre, University of Washington, Seattle.

The intraperitoneal injection of multiple, spaced, sensitizing doses of diluted egg-white or rabbit serum serves to sensitize white mice so that anaphylaxis is fatal when the shocking dose is administered either intravenously or intraperitoneally. Fatal anaphylaxis was produced as early as the 12th day after the beginning of the sensitization treatment and the 4th day following sensitization. It persisted rather uniformly until the 15th or 20th day, when a drop in sensitivity occurred. By the 30th day fatal shock was produced in only an occasional animal, and by the 45th day anaphylaxis was absent. A single large dose of egg-white was not efficient for anaphylactic sensitization. Anaphylaxis was not as constant among very young mice as among older animals. Repeated sub-lethal doses of antigen served to desensitize actively sensitized mice. Fatal anaphylaxis was not produced in mice previously treated with the sera of sensitized rabbits or mice. In a second phase of the work, adrenalectomized mice were used. Such mice were extremely susceptible to shock, yet they could be desensitized. Although there was an apparent correlation between anaphylaxis and serum precipitins during the early period following sensitization, fatal anaphylaxis was often produced months later, when precipitins were not demonstrable. Passive sensitivity produced with rabbit serum was of short duration and usually disappeared by the 30th day.

M48. Incidence of H and O Agglutinins for Typhoid and Paratyphoid Bacilli in the Sera of Persons with no History of Enteric Disease. John Phillips and H. J. Sears, University of Oregon Medical School, Portland.

More than 200 specimens of serum from individuals with no history of enteric disease have been examined for the O and H agglutinins of the typhoid bacillus and of two of the paratyphoid bacilli. Seventy per cent of the sera showed no agglutination with any antigen in a dilution of 1:20, while 30 per cent were positive for one or more of the antigens at this or higher dilutions. The incidence of agglutination was higher for the O antigens than for the H, and agglutinins were more frequently demonstrated for the paratyphoid B organism than for typhoid or paratyphoid A bacilli. A small group of sera from vaccinated persons showed variable results.

M49. Some Serological Relationships of the S, R and G Phases of Bacillus salmonicida. D. C. B. DUFF, University of British Columbia, Vancouver, Canada.

Cultural phases S, R and G of *Bacillus salmonicida* have been established previously by the author (1937). Subsequent work has attempted to elicit the serological relationships of these phases, both within homologous cultures and among heterologous strains. Methods so far have been confined to reciprocal agglutinin absorptions and to precipitation reactions. Antigen has always been killed with 0.2 per cent formaldehyde, and suspensions for agglutination titration have been made in 0.05 per cent sodium chloride. Invariably R antigen showed a much lower absorptive capacity, both for R and for S agglutinin, than did S strains.

In each of 14 *Bacillus salmonicida* strains, reciprocal absorptions showed antigenic composition of the form $R = S + n$. In 5 other strains, $S_1 = R_1 + n_1$. With all 19 strains, the G phase possessed some antigens common to both R and S, together with some individual antigen. Comparison of R phases of different strains showed slight antigenic differences, in some cases. A more marked variation occurred among the S phases of the same strains. Antigenic variation in S strains, as determined by the experimental methods noted, could not be correlated with virulence. Sixteen morphologically true single-cell S strains were pathogenic to goldfish, but possessed varying virulence, 3 strains being non-pathogenic. No R or G phase of any strain ever produced disease. Precipitation reactions in general support the agglutinin-absorption findings.

M50. Further Studies on the Purification and Analysis of Diphtheria Toxin. AUGUSTUS B. WADSWORTH AND MARY W. WHEELER, New York State Department of Health, Albany.

Previous papers have described the production of toxin in a purely synthetic medium and its purification by ultrafiltration. The present report will record the results of further studies on the preparation of toxic and non-toxic fractions from synthetic medium, and on the purification of toxin by ultrafiltration. Investigations of the biological, chemical, and physical properties of these fractions will be described.

M51. A Study of the Quantitative Complement-Fixation Test in Tuberculosis. AUGUSTUS B. WADSWORTH, ELIZABETH MALTANER AND FRANK MALTANER, New York State Department of Health, Albany.

The complement-fixation test with tubercle antigen has been completely revised to provide a quantitative titration of the activity of the serum. The new procedure is based on linear relationships which have been demonstrated in previous studies between complement and serum or antigen, when all three reagents are used in amounts to give maximal interaction. The present paper will record the results obtained in quantitative tests of specimens from active and inactive cases of tuberculosis in various stages of advancement.

M52. Complement-Fixation Reactions with Pertussis Undenatured Bacterial Antigen. H. M. POWELL AND W. A. JAMIESON, Lilly Research Laboratories, Indianapolis, Ind.

In view of the importance of the native-protein content of Pertussis Undenatured Bacterial Antigen, work was undertaken to correlate biological methods of testing with chemical standardization. Positive complement-fixation results were observed to be specific with the antigen, and appear to be useful in assaying the native-protein content as determined by chemical methods. Such biological tests necessitate but a single day for testing as compared to a period of 6 or 8 weeks for conducting mouse tests.

M53. Treatment of Antiserum to Remove Serum-Sickness-Causing Factor. LLOYD R. JONES AND MOYER S. FLEISHER. St. Louis University School of Medicine, St. Louis, Mo.

Phenolized and non-phenolized portions of a lot of tetanus antitoxin (whole horse serum) were subjected to various modifications of a method for the removal of the factor causing serum sickness in the rabbit. The method involved the addition of dilute alkali to the serum, application of heat at 50° or 55°C. to the mixture for one or one and one-half hours, and subsequent neutralization with an equivalent amount of acid. After restoring the diluted serum to its original volume by evaporation, it was tested in rabbits for serum-sickness-causing activity and titrated for antitoxic content by protection tests in guinea pigs against known doses of toxin.

The effect of varying the concentration of alkali, the temperature, and the period of application on the removal of serum-sickness-causing factor and on the destruction of antitoxic activity will be described. In dealing with the sample of non-phenolized serum, it was found possible to adjust the method to the particular requirements of the lot of serum under examination in such a way as to remove all of the serum-sickness-causing activity and yet to retain 80 per cent of the original antitoxic content. Phenolized serum, however, required somewhat more drastic treatment (*e.g.*, higher concentrations of alkali) for the removal of serum-sickness-causing activity. Consequently, there was a somewhat greater destruction of antitoxin.

M54. The Protective Action of Egg-Albumen when Administered Orally with Clostridium botulinum (Type C) Culture to Pigeons. MILLARD F. GUNDERSON, University of Nebraska College of Medicine, Omaha.

The toxic potency of the culture of *Clostridium botulinum* (Type C) employed in these studies was such that 0.001 cc. injected into each of 20 pigeons caused their death within 24 hours. When fed to 10 birds in 1 cc. doses, this culture killed 3 within 9 hours, 3 within 18 hours, and 1 within 36 hours. One pigeon was sacrificed while lethargic after 144 hours and 2 displayed no symptoms. When 3 cc. amounts of this culture were fed to each of 10 birds, all 10 died within 24 hours.

Birds were fed 3 cc. doses of culture mixed with 0.5 cc. amounts of proteins of various kinds. Antitoxins and normal sera had a detoxifying effect. In 3 instances the birds survived. In the case of a single bird death was delayed for 12 days. Egg-albumen in amounts of 0.5 cc. was mixed with each 3 cc. dose of culture and this mixture was fed to each of 10 birds. One survived, and the rest lived for periods varying from 8 to 15 days.

The ingested toxin-protein complex could be broken up by drenching the birds with 2 cc. amounts of a 5 per cent sodium carbonate-saturated sodium sulfate solution. A series of 10 poisoned birds so treated showed a shortening of the period between dosage and death. Four died within 24 hours, 2 within 12 hours, and 2 lived for 7 days.

Gelatin and heated serum gave only indifferent results. In similar experiments with Type A toxin little evidence of binding was secured.

The relation of these observations to naturally occurring avian botulism is considered.

M55. *The Susceptibility of the Antigenic Types of Staphylococci to Several Protective Enzymes.* RICHARD THOMPSON AND EDUARDO GALLARDO, JR., College of Physicians and Surgeons, Columbia University, New York City.

In a previous communication we reported that pathogenic staphylococci of Type A are able to grow in human whole blood and serum to a much greater extent than are the other types. In view of the suggested relationship between pathogenicity and resistance to protective enzymes, the susceptibilities of the different types of staphylococci to several agents have been compared. Purified egg-white lysozyme, human tears, rabbit serum and rabbit-leucocyte extracts have been used. The tests were done by comparing the concentrations of the various agents required to produce visible lysis of suspensions of the different organisms and to inhibit their growth in broth or on agar. *Micrococcus lysodeikticus* was very susceptible to all of the agents. Of the staphylococci, Types B and C showed definite degrees of visible lysis by egg-white lysozyme, tears and leucocytic extracts. Serum inhibited the growth of all but the Type A staphylococci which were affected only by leucocytic extracts. However, they were markedly inhibited by these extracts. It is uncertain to what extent the differences in the agents are qualitative rather than quantitative; but it is indicated that the leucocytic agent, at least, is qualitatively different from egg-white lysozyme.

M56. *Proteinases and Peptidases during Various Phases of Experimental Tuberculosis in Rabbits.* CHARLES WEISS AND A. KAPLAN, Mount Zion Hospital, San Francisco, Calif.

Employing hemoglobin as a substrate, it was shown that polymorphonuclear neutrophilic leucocytes, monocytes and epithelioid cells contain a cathepsin with optimal activity at pH 3, (range pH 1 to 5.5); also a dipeptidase hydrolyzing alanyl-glycine at pH 8. Carboxypeptidase splitting chloracetyl-tyrosine at pH 8 is lacking. The monocytes may, however, be differentiated from myelocytic leucocytes, since only the latter can split alanyl-glycine at pH 5.5 and hydrolyse gelatin and casein at pH 8. Since epithelioid cells are derived embryologically from monocytes, it is of interest to note that they have an enzyme-pattern similar to the latter, but not to polymorphonuclear neutrophilic leucocytes.

Tissues of rabbits (normal, allergic and immune) were examined for their proteinase and peptidase content. The data will be presented and discussed.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. Distribution and Activity of Azotobacter Organisms in Arizona Soils.

W. P. Martin and R. A. Ayers, College of Agriculture and Agricultural Experiment Station, University of Arizona, Tucson.

Some 76 cultivated soils of Arizona have been examined for the presence of *Azotobacter* organisms. Only 9 samples did not contain the bacteria; these had a total salt content of over 3,000 p.p.m. The bacteria were very active in these soils, as indicated by an average fixation of over 22 mg. of nitrogen per gm. of soil tested. Curie's method was used in these tests and the incubation period was only 4 days. The amount of nitrogen fixed per gm. by *Azotobacter* organisms from some Iowa soils, in which a highly significant increase in the growth of timothy resulted from an inoculation with a vigorous growth of the organisms after the soils had been improved with lime, was but 12 mg. of nitrogen per gm. of soil tested. Consequently, in the cultivated soils of Arizona the amount of nitrogen fixed by *Azotobacter* organisms may aid materially in the maintenance of the soil's nitrogen supply and may help to account for the large amount of nitrate-nitrogen found in the drainage waters: this has exceeded 300 p.p.m. in some cases.

The range soils of Arizona were generally lacking in *Azotobacter* organisms, the bacteria having been found in only 6 of 23 samples tested. The amount of nitrogen fixed per gm. in these samples averaged about 3 mg. per gm. of soil tested, which indicates that the bacteria in these samples were not very active as compared with those in the cultivated soils.

A2. Some New Non-Symbiotic Nitrogen-Fixing Bacteria from the White Sands of New Mexico.

Charles B. Lipman, University of California, Berkeley.

In a survey of the microbial flora of the White Sands of New Mexico, which are an almost pure gypsum formation with shifting dunes, it was discovered that the sands inoculated into appropriate media yielded marked fixation of nitrogen. In hundreds of examinations it has been impossible to find any definite *Azotobacter* organisms in the flora responsible for the fixation. Therefore, a special study covering a period of two years has been made of the nitrogen-fixing bacteria occurring in the White Sands. As a result of these studies several forms of bacteria have been discovered which seem to be different from any non-symbiotic nitrogen-fixing bacteria thus far described in the literature. A brief account of these new forms and of their nitrogen-fixing powers will be given in the paper.

A3. The Activity of Azotobacter Organisms under Field Conditions. S. C. VANDECAVEYE, State College of Washington, Pullman.

Using the silica-plate method, periodical counts of *Azotobacter* organisms were made for several years on representative soil samples obtained fresh from the field. A marked seasonal fluctuation in the numbers of *Azotobacter* organisms and a gradual disappearance of these bacteria from certain soils which once supported an active flora were observed.

Berkefeld-N-candle filtrates were obtained from soil added to an *Azotobacter*-nutrient solution and incubated at 28°C. for 36 hours. These had an inhibiting effect on the growth of *Azotobacter* organisms on agar plates when proper amounts of the filtrates were applied to 24-hour liquid cultures of *Azotobacter* strains. The inhibiting effect was expressed in the form of plaque-like formations. A few attempts to demonstrate transmissible lysis by serial transfers of a limited number of strains of *Azotobacter* were unsuccessful, but inoculation of the affected soil with different species and strains of this organism disclosed a marked difference in the tolerance of various strains to the inhibitng factor in the soil.

A4. Is the Ability to Metabolize Atmospheric Nitrogen a Constant Characteristic of Azotobacter Organisms? P. L. GAINEY, Kansas State College, Manhattan.

A limited percentage of strains of *Azotobacter chroococcum*, when grown for long periods on an agar medium high in nitrate-nitrogen, will lose the ability to grow on a "nitrogen-free" agar. Certain of these strains, when supplied with limited quantities of fixed nitrogen, rapidly regain the ability to grow upon "nitrogen-free" agar. Other strains seem to lose permanently the ability to grow upon a "nitrogen-free" agar, all subsequent growth being directly proportional to the quantities of fixed nitrogen supplied in the medium. Failure to grow upon a "nitrogen-free" agar is due apparently to an inability to metabolize free atmospheric nitrogen.

A5. Strain Variation and Host Specificity of Rhizobium within the Cowpea Cross-Inoculation Group. O. N. ALLEN AND ETHEL K. ALLEN, University of Hawaii, Honolulu.

A study has been made of the infective and effective abilities of 54 strains of *Rhizobium* isolated from 28 species of leguminous plants when used as inocula for 20 plant members of the cowpea cross-inoculation group. Results have been based upon three replicated greenhouse experiments which were conducted under approximately the same meteorological conditions.

Marked differences were noted in the responses of the plants to the various inocula. Only 25 per cent of the strains produced nodules upon all of the test plants. In regard to benefits derived from the inoculation, 48, or 89 per cent, of the strains were beneficial upon more than 10 of the species of plants. Four strains benefited the growth of 19 species. Ten of the test plants were nodulated by all of the inocula. *Vigna sinensis* was the most susceptible, inasmuch as each strain of *Rhizobium* not only produced nodules, but enhanced its growth; while the other extreme was represented by *Phaseolus lunatus* upon which only 22 strains produced nodules, none of which were beneficial.

The results suggest that variations in the infectiveness and effectiveness of *Rhizobium* strains from plants of the cowpea group may be of a greater magnitude than those previously recorded for the other cross-inoculation groups.

A6. A Cytological and Histological Study of the Root-Nodules of the Peanut (Arachis hypogaea L.). O. N. ALLEN AND ETHEL K. ALLEN, University of Hawaii, Honolulu.

Inoculation of peanut plants with good and poor strains of nodule-forming bacteria has shown two types of nodules: one, comparatively large, beneficial nodules located on the upper central tap-root system; the other, tiny, innumerable, non-beneficial nodular swellings, scattered throughout the root-mass, and most readily seen when immersed in water. Both types were located in the axils of the roots.

Results obtained do not indicate any differences in the origin of these nodular types, inasmuch as each arises apparently from infection within the ruptured axillary tissue at the point of emergence of the lateral roots. Paraffin sections of the infected areas made prior to a macroscopic enlargement of the nodular tissue show a distinct proliferation of cells originating opposite protoxylem strains in the axils of the lateral roots. Such proliferations are at first completely enclosed within the pericycle of the roots. In the early stages of formation the nodular swelling is entirely meristematic; later a ring of endodermis is formed, and as growth progresses a periderm layer encloses the rapidly growing nodular mass. The parenchymatous cells of the root-cortex appear torn and partially absorbed due to the outward growth of the nodular tissue, but are retained in later stages as a sheath distinct from the nodular cortex. It appears that these nodules arise endogenously from the same layers of cells as do lateral roots. The bacterial phase of the *Rhizobium* organisms within the nodules presents morphological variations different from those heretofore described in nodules of other species.

A7. Combination of Mixed Groups of Rhizobium in a Single Culture. V. S. Bond, Kalo Inoculant Co., Quincy, Ill.

Cultures of root-nodule bacteria are commonly prepared with organisms of but one plant group, *i.e.*, the culture is applicable only to those leguminous seeds with which a single type of *Rhizobium* will cross-inoculate. In practice, as many legume inoculants must be prepared and distributed as there are common cross-inoculation groups. The desirability of combining the organisms representative of two or more groups in a single unit led to the present investigation.

Combination cultures were checked according to standard experimental technic by growing the inoculated plants in sterile sand supplemented with all of the essential nutrients except nitrogen to determine whether they satisfied the requirements of a satisfactory single-group culture. These requirements demand that, at any time within the expiration date, sufficient organisms of the proper type be present to produce an adequate nodulation and a high nitrogen-fixing efficiency to yield maximal benefit to the host plants. Nodule formation was checked after growth for 3 or 4 weeks and the ability of the organisms to fix atmospheric nitrogen was measured by analyzing full-grown plants for total-nitrogen content. In every experiment, uninoculated controls to detect possible contamination and controls inoculated with standard single-type culture to afford direct comparison were included.

The data indicate that practical legume inoculants containing two or more culture groups combined in a single unit may be prepared which compare favorably with standard single-group cultures, with respect to both nodule formation and efficiency of nitrogen fixation.

A8. Growth-Factor Requirements of Rhizobium trifolii. P. M. West and P. W. Wilson, University of Wisconsin, Madison.

Studies on the growth-factor requirements of *Rhizobium* strains indicate that the heat-stable substance extracted from *Azotobacter* cultures and described by Allison and Hoover provides the nodule organism with a constituent greatly increasing the initial rate of growth. Although this material is capable of inducing growth in a medium of unfavorable oxidation-reduction potential, it appears that its stimulative value is greater than that which could be obtained by the simple addition of reducing substances. While effective in accelerating the rate of cell multiplication, it is not indispensable to growth as was previously believed.

Although *Rhizobium* strains do not appear to synthesize this heat-stable stimulative material found in *Azotobacter* or yeast extracts, further studies have revealed that a more active, heat-labile substance is produced by the organisms themselves when growing in a purely synthetic medium. Even normal loop transfers carry sufficient of this factor from the previous culture to allow a satisfactory growth to occur in a medium of the proper oxidation-reduction potential. Through special precautions it has been possible to prepare inocula relatively free from the old-culture material. This has resulted in a marked decrease or in a complete failure on the part of the cells to multiply. By suspending such an inoculum in an unheated cell-free filtrate from a growing culture, however, growth takes place normally. The stimulative effect of small amounts of unheated culture-filtrate on the growth of the organism has also been observed by a giant-colony technic modified for quantitative studies.

A9. The Presence and Significance of Oxalacetic Acid in Plant Tissues.

ORVILLE WYSS AND P. W. WILSON, University of Wisconsin, Madison.

Recently, it has been suggested that oxalacetic acid may play an important intermediary rôle in biological nitrogen fixation. The occurrence of the acid in leguminous plants actively fixing nitrogen has been used in support of this hypothesis. The established importance of oxalacetic acid in the four-carbon dicarboxylic acid cycle of animal-tissue respiration suggests the importance of a study of these acids in plant tissue.

Oxalacetic acid was determined in grasses and cereals and other types of non-leguminous plants. Legumes which were fixing nitrogen and others supplied with combined nitrogen were compared as to content of the keto-acid. Determinations were conducted by both a colorimetric and a manometric method. The results are presented and interpreted in the light of their significance for the biochemistry of biological nitrogen fixation.

A10. The Influence of Cropping on the Nitrogen-Fixing Capacities of the Soil. J. E. GREAVES AND A. F. BRACKEN, Utah Agricultural Experiment Station, Logan.

When soils from wheat land and adjacent virgin land from Juab and Cache Counties, Utah, were inoculated into a synthetic medium, the gains in nitrogen from the cropped soils were greater than from the virgin soils. The nitrogen gains made by the Cache soils were considerably greater than those of the Juab soil. This was due to two factors: (1) the Cache soils contain a larger quantity of carbonaceous material than the Juab soils; (2) practically all of the Cache soils carry a rich *Azotobacter* flora, whereas these organisms are generally absent in the Juab soils. When alfalfa soil was inoculated into the same medium, the fixation was about twice that observed when virgin or wheat-land soil was used. The increase was manifested in the first-, second-, and third-foot sections of soil and was the result of either a direct or indirect action of the legume upon the non-synthetic nitrogen-fixing organisms.

The addition of ground alfalfa, ground pea vines, and ground straw to the Nephi dry-farm soil increased the nitrogen-fixing capacity during the first two years to approximately the same extent. All of the plant residues caused greater increases in the nitrogen in the soil than could be accounted for by the added nitrogen; hence the increases must have been due to greater nitrogen fixation from furnishing energy to the non-symbiotic nitrogen-fixing organisms. The greatest gains in soil nitrogen were caused by the addition of alfalfa. Some of the added carbonaceous material was still present after eleven years.

A11. Nature of Hydrogen Inhibition of the Symbiotic Nitrogen-Fixation Process. SYLVAN B. LEE AND P. W. WILSON, University of Wisconsin, Madison.

It has been demonstrated by Wilson and his associates that molecular hydrogen is a specific inhibitor for the symbiotic nitrogen-fixation process by root-nodule bacteria and red clover plants (*Trifolium pratense*) During the past year, experiments have been conducted in an attempt to determine the nature of the inhibiting action of hydrogen. In these experiments the pN_2 and pH_2 of the atmosphere furnished to inoculated red clover plants were varied. In some experiments, the pN_2 was held constant at partial pressures at which it is not a limiting factor in the nitrogen-fixation process (pN_2 0.2 atm. or above) and the pH_2 was changed. In other experiments, the pH_2 was held constant at partial pressures at which it was previously found to exhibit inhibiting action (0.2 to 0.6 atm.) and the pN_2 was varied.

Mathematical analyses of the results (total nitrogen fixed) obtained from such experiments to date indicate that hydrogen is acting as a competitive inhibitor in the symbiotic nitrogen-fixation process.

A12. Some Effects of Benzoic Acid Compounds on Azotobacter Organisms.

HERBERT W. REUSZER, Colorado Agricultural Experiment Station, Fort Collins.

Experiments were performed to test the effect of various benzoic acid compounds upon the flora of the soil, particularly upon the population of *Azotobacter*. The presence of these compounds brought about a great increase in the abundance of *Azotobacter* organisms. It was found, further, that benzoic acid and sodium benzoate when present in proper concentration caused not only a quantitative but also a qualitative difference in the population of *Azotobacter*. When these compounds were added to a soil in a concentration of 1 per cent, *Azotobacter chroococcum* appeared to be the only species of *Azotobacter* present. When the concentration of the compounds was increased to 2.5 per cent, a different strain of *Azotobacter* appeared which at subsequent periods of incubation displaced *Azotobacter chroococcum* entirely. The new strain was a highly motile organism forming a soluble green pigment. Its identity has not yet been definitely established, but it does not appear to have been described previously from American soils. In artificial media, the new strain is capable of growing in higher concentrations of sodium benzoate than is *Azotobacter chroococcum*. Preliminary experiments indicate that it is capable of fixing atmospheric nitrogen in the presence of considerable quantities of combined nitrogen.

A13. Factors Influencing the Bacterial Content of Aseptically Drawn Milk.

KENNETH R. STEVENS AND LEWIS W. JONES, Utah State Agricultural College, Agricultural Experiment Station, Logan.

The occurrence of bacteria in samples of certified milk prompted an investigation designed to answer the following questions. What count of bacteria is really unavoidable under aseptic conditions of production? Are cows, quarters, or months significant factors in accounting for bacteria in milk produced under these conditions? What influence do these factors have on the types of bacteria present?

Samples of milk, taken from 10 healthy cows over a four-month period, were properly diluted and plated on a modified standard agar. Six replicates and a check were made on the sample of milk obtained from each quarter. Incubation was carried on at 30°C. for 5 days, followed by 2 days at 37°C. The average count per cc. of milk from each quarter of the 10 cows for the four-month period was: left front, 210; right front, 365; left rear, 157; and right rear, 224. The average counts for the individual cows over the period varied from 49 to 511.

The data were analyzed by Fisher's variance method to determine the influence of the variables on the total number and types of bacteria found. Based on the four-month average, the count showed a highly significant difference between cows, between quarters, and in the interaction of cows and quarters. The interaction between cows and months was somewhat significant, but that between months and the interaction of quarters and months was not significant. The percentage of cocci present was significant only between cows and in the interaction between cows and months.

A14. Lactobacillus thermophilus in a City Milk Supply. David B. Charlton and George F. Steel, Oregon State College, Corvallis.

In view of the occurrence of unusually high bacterial counts in the milk from certain pasteurizing plants, a study was made of the thermophilic and thermoduric bacteria found in the pasteurized milk (45 milk plants) of Portland, Oregon. Isolations of bacteria from the pasteurized milk were made, and the resistance of the organisms to heat was determined in the laboratory.

Lactobacillus thermophilus was most frequently found to be responsible for the high bacterial counts noted. It occurred more or less regularly in milk from 11 pasteurizing plants. The "Standard Methods" plating technic, as employed in the city for routine control work, does not allow a good growth of the organism to take place. *Lactobacillus thermophilus* has a growth-temperature range from 36° to 62°C. The colony is not "pin-point" in size, as originally reported, when the media contains either proteose or tryptone peptone in the usual 0.5 per cent concentration. The peculiar growth requirements of *Lactobacillus thermophilus* and the fact that in various types of cultures numbers of viable cells diminish rapidly may explain why few investigators appear to have isolated it.

The organism has been isolated by one of us (Charlton) from samples of pasteurized milk obtained in two other cities. In each case, a slight "off" odor in the milk was responsible for attention being directed to it.

A15. A Differential Stain for the Direct Examination of Milk. JEAN BROADHURST AND CHARLES PALEY, Teachers College, Columbia University, New York City.

With the stain described below, bacteria and white corpuscles are blue, the milk background is faintly pink or colorless. Higher bacterial counts are obtained than with the usual methylene blue stains (Breed, Newman) and less eye-strain is experienced.

The preparation of the stain is as follows. Dissolve from 0.6 to 1 gm. of methylene blue in 200 cc. of 70 per cent alcohol. Add 5 cc. of 1 per cent basic fuchsin (1 gm. dissolved in 100 cc. of 95 per cent alcohol). Add 3 cc. of aniline and shake well. Add 12 cc. of dilute sulfuric acid (5.7 cc. of concentrated sulfuric acid, c.p., in about 90 cc. of distilled water, then made up to 100 cc.). Mix well and filter. To every 100 cc. of filtrate, add 50 cc. of distilled water and shake well. (The directions must be followed closely. If the stain thickens on adding the sulfuric acid, dilute it with distilled water before, instead of after, filtering.)

The staining procedure is as follows. Make milk smears on glass slides as usual, 1/100 cc. of milk being spread over 1 sq. cm. (or preferably 2 sq. cm.) When dry, immerse in xylol for 1 min. and drain dry. Immerse in 95 per cent ethyl alcohol for 1 min. and drain dry. Dip slowly, 3 times, in the stain (or flood slide with stain for ½ min.) and drain dry. When thoroughly dry, rinse in water until the blue color is washed out and the smear becomes pink. Dry and examine with the oil immersion lens as usual.

A16. The Rôle of Agglutinins in the Hotis Test. ERNEST C. MCCULLOCH, State College of Washington, Pullman.

The appearance of typical yellow balls or flakes on the side of a tube of milk following incubation in the presence of 0.025 per cent of bromcresol purple has been considered diagnostic of *Streptococcus agalactiae*. We failed to obtain typical reactions with sterilized bromcresol purple milk and pure cultures, although aseptically drawn negative milk from most cows supported typical Hotis reactions with pure cultures as did sterilized milk after the addition of small amounts of most blood sera. Agglutinin absorption markedly reduced the titer for the strain used and both milk and milk-serum mixtures supported the test after heating to 65°C., but not after 75°C., which corresponds to the thermal tolerance of agglutinins.

Our data indicate that Hotis-positive reactions will be produced by any organism which: (1) multiplies in the udder and is present in the milk drawn; (2) stimulates the production of agglutinins; (3) tolerates 0.025 per cent of bromcresol purple; (4) forms clumps, when grown in the presence of its agglutinins; and (5) produces sufficient acid from lactose to increase the hydrogen ion concentration of the clump to *circa* pH 5.4.

Hotis-positive organisms were obtained from the nostrils of Hotis-negative cows and from the throats of 76 of 84 students. The streptococci from different positive reactions or from different clumps in the same tube often differed serologically and physiologically. The technic provides a convenient method for isolating streptococci from contaminated sources, since mixed cultures of streptococci tend to segregate into homologous serological groups during agglutination by sera containing agglutinins for several groups.

A17. The Effect of Soaps on Streptococcus agalactiae. ERNEST C. MCCULLOCH, State College of Washington, Pullman.

Soaps and especially the newer types of household detergents are effective disinfectants for *Streptococcus agalactiae.* The relationship between the chemical and physical characteristics of these products and their germicidal efficiencies, coefficients of dilution and temperature coefficients are shown by graphs and charts.

A18. A Simplified Method for the Bacteriological Examination of Water. JAMES E. WEISS AND CHARLES A. HUNTER, South Dakota State Health Laboratory, Vermillion, Brooklyn College, Brooklyn, N. Y., and Kansas Public Health Laboratory, Topeka.

A simplified method for determining the potability of water has been developed and compared with the standard method. The new or "bottle" method consisted of adding 5 cc. of a concentrated broth (24 gm. of nutrient broth (Difco) to 100 cc. of water) to the sample bottle containing approximately 150 cc. of water, thus making 155 cc. of nutrient broth. After adding the concentrated broth to the water in the bottle, the mixture was incubated at 37°C. over night. The next day a loopful of broth from the bottle was streaked on a plate of eosin-methylene blue agar. Confirmation was carried out in the usual manner.

The "bottle" method was compared with the standard method using 638 water samples. It was found that 29.6 per cent of the samples gave positive results by both methods, that 60.2 per cent were negative by both methods, and that there was disagreement in 10.2 per cent. Of the 65 water samples showing disagreement, the standard method was positive and the "bottle" method was negative in 1.9 per cent of the samples, and the standard method was negative and the "bottle" method was positive in 8.3 per cent of the samples. Thus, the difference in favor of the "bottle" method was 6.4 per cent.

Sterile bottles containing the required amount of concentrated broth were sent into the field for trial with a number of samples of water. The results were excellent. The "bottle" method proved to be much more rapid and yielded fewer false reactions. Thus, while being more efficient, it was simpler and more economical.

A19. Effect of Temperature and Test Reagents on the Voges-Proskauer and Methyl Red Reactions. REESE VAUGHN, NANCY MITCHELL AND MAX LEVINE, Iowa State College, Ames.

Of 221 strains of the colon group tested for the production of acetyl-methyl-carbinol by the 10 per cent KOH method, only 21 (9.5 per cent) were positive after one day and 26 (11.8 per cent) after two days of incubation at 37°C., as contrasted with 45 (20.4 per cent) and 48 (21.7 per cent) giving positive reactions at corresponding periods when the temperature of incubation was 30°C. The creatine-KOH reagent gave many more positive reactions. Thus, 38 (17.2 per cent) and 42 (19 per cent) were positive after one and two days of incubation, respectively, at 37°C. and 51 (23.1 per cent) after one day at 30°C. The α-naphthol reagent was most sensitive, yielding 44 (19.9 per cent) positive Voges-Proskauer tests after one day of incubation at 37°C. and 51 (23.1 per cent) after one day at 30°C. Of 198 Voges-Proskauer-positive strains tested with the standard KOH, the creatine-KOH, and the α-naphthol reagents, the positive reactions obtained one-half hour after addition of the reagents were 0.5 per cent, 52 per cent and 94.5 per cent. These figures rose to 10.7 per cent, 61.6 per cent and 99.5 per cent, respectively, after contact for one hour.

The technics recommended in "Standard Methods of Water Analysis" for the Voges-Proskauer reaction (24 to 48 hours at 37°C. with 10 per cent KOH as the test reagent) and for the methyl red test (3 to 4 days at 37°C.) are not suitable for the detection of the maximal number of Voges-Proskauer-positive or methyl red-negative strains, and tend to obscure the correlation of these reactions. A temperature of incubation of 30°C. and the use of the Barritt α-naphthol reagent are recommended.

A20. Dye Modifications of Bismuth-Sulfite Media. HERMAN C. MASON, University of Illinois College of Medicine, Chicago.

The problem was to determine the action of dyes and certain other substances on the bismuth-sulfite medium of Blair and Wilson in order to provide supplementary procedures to those now in use for the differentiation of certain Gram-negative enteric bacilli from each other.

The following media were used: (1) Wilson and Blair's; (2) Tabet's modification of Wilson and Blair's; (3) bismuth sulfite (Difco); and (4) selenite enrichment. The dyes and other substances added to the first three of these media, in concentrations ranging from 0.003 to 0.15 per cent, were as follows: acid acriflavine, neutral acriflavine, acid fuchsin, basic fuchsin, fuchsin, brilliant cresol blue, chrysoidin, Congo red, fluorescein, indigo carmine, malachite green, methylene blue, oxyquinoline sulfate, phenol red, phosphomolybdic acid, pyronin, quinaldine red, scarlet red, sodium hippurate, tannic acid, thionin and trypan blue. A large number of enteric bacilli chosen from a variety of genera and a few other kinds of bacteria were used as test organisms. Broth and agar-slant cultures from 18 to 24 hours old of freshly isolated and old laboratory strains were employed.

The addition of the dyes produced distinctive colonies for the typhoid and paratyphoid strains. The most favorable reaction was secured by adding phosphomolybdic acid in a concentration of from 0.003 to 0.005 per cent to the bismuth-sulfite medium. The addition of such substances as tannic acid, sodium hippurate and phenol red can be adjusted to allow growth of the dysentery bacilli to take place, but this will also permit the development of other organisms.

A21. Studies on the Flora of North Pacific Salmon. JANE E. SNOW AND PAUL J. BEARD, Stanford University, Calif.

Bacteriological samples from live salmon, from salmon stored in canneries before canning, and from the sea, from the mouth of the Columbia River at Astoria, Oregon, to the upper end of Cook Inlet, Alaska, have been investigated. Six genera, *Achromobacter*, *Pseudomonas*, *Micrococcus*, *Flavobacterium*, *Kurthia* and *Proteus* have been isolated consistently from all of the sources examined. The potential significance of these organisms in initiating changes leading to more advanced decomposition is discussed. The coliform group was found only in samples which had been subject to land contamination.

A22. *Some Types of Microörganisms Isolated from Whole-Kernel Sweet Corn prior to Canning.* MATTHEW E. HIGHLANDS, University of Maine, Orono.

A study has been made of the types and numbers of microörganisms occurring on freshly cut whole-kernel sweet corn. Samples were taken at various stages during production up to the actual canning. In addition, the cut corn was held for several hours at atmospheric temperature, during which period representative portions were plated to detect changes in the microbial population. The samples were plated on corn-extract agar and incubated at 30°C. and at 55°C. An examination of the material for the presence of anaerobic thermophilic bacteria was made. The predominant types were isolated from the plates and identified. Of the 61 cultures isolated, the following genera and species were identified: *Achromobacter globiforme*, *Micrococcus conglomeratus*, *Serratia rubra*, *Serratia rosea-alba*, *Sarcina flava*, *Phytomonas stewarti*, *Aerobacter sp.*, *Achromobacter sp.*, *Pseudomonas sp.*, *Phytomonas sp.*, *Flavobacterium sp.*, *Chromobacterium sp.* All of the cultures except the last were identified according to the system employed in Bergey's Manual. No thermophilic organisms were encountered in any of the samples examined.

A23. *Spore-Forming Anaerobes Causing Spoilage in Canned Acid Foods.* C. T. TOWNSEND, University of California, San Francisco.

In recent years a number of cases of spoilage in tomatoes and low-acid fruits, such as pears, figs and nectarines, has been found to be due to a group of spore-forming anaerobes. The rods are somewhat short, with terminal oval spores. They are non-motile. The optimal temperature for growth is 30°C., but development may occur slowly at 37°C. Subsurface colonies in agar are lenticular. There is no liquefaction of gelatin. Growth with the production of some gas, but not of acid or clot, occurs in litmus milk. Indicators are reduced. The group is markedly saccharolytic, but apparently cannot utilize lactose. A considerable amount of volatile acid is produced, and the gas liberated contains approximately as much hydrogen as carbon dioxide. Growth has been noted in pear juice down to pH 3.55.

Heat-resistance studies of spores in neutral phosphate buffer and in fruit juices, using 20,000 spores per container, have shown maximal thermal-death times at 100°C. of 40 min. in phosphate-buffer; of 20, 12 and 6 min., respectively, in tomato juice of pH values 4.5, 4.1 and 3.8; of 20 and 12 min. in pear juice of pH 4.5 and 4.1; and of 10, 7 and 7 min. in apricot juice of pH 4.5, 4.15 and 3.95. Slopes of the thermal-death-rate curves (z values) have been obtained.

Use has been made of spore suspensions of these organisms in inoculating experimental packs of tomatoes and fruit juices to determine processes necessary for sterilization.

A24. The Correlation of Resazurin-Reduction Rates and Plate Counts as Indices of the Bacterial Condition of Fresh and Frozen Foods

B. E. PROCTOR AND DAVID G. GREENLIE, Massachusetts Institute of Technology, Cambridge.

A series of comparative determinations of bacterial plate counts and resazurin-reduction rates was conducted on fresh and frozen foods, including hamburg steak, fish, crabmeat, eggs and vegetables, for the purpose of determining whether the use of redox-potential indicators offers a relatively accurate and prompt evaluation of quality.

The procedure for the preparation of the resazurin-test samples, concentrations of reagents, and the incubation temperature may be varied to advantage with different foods. Evacuation of the tubes used for the resazurin-test was found to be desirable. Filtration of certain of the food solutions to remove large suspended particles tended to increase the time necessary for reduction but appeared to give more consistent results.

Foods having high bacterial plate counts, *i.e.*, over a million per gm., had reduction rates such that color changes of the dye could usually be noted within from 3 to 5 hours of incubation. In the case of foods with very high plate counts, the reduction was sometimes almost complete in ½ hour.

The results indicate the possible application of a modified resazurin method by control officials and others interested in the detection and segregation of foods having abnormally high bacterial contents, or foods which have been improperly handled or stored.

A25. Concerning the Genera of Yeasts Occurring on Grapes and Grape Products in California.

E. M. MRAK AND L. S. McCLUNG, University of California, Berkeley.

With the exception of brief studies by Holm (1908), and by Cruess (1918), the literature on the ecology and taxonomy of yeasts in California exists only as occasional references in scattered papers. The present survey was undertaken to increase existing knowledge of the occurrence and distribution of the various yeasts occurring on grapes from the principal vineyard districts of California. The study also included an investigation of the yeasts found in wines, during various stages of fermentation, which were fermented under natural conditions during the 1936 vintage. Two hundred and thirty-one cultures, representing 119 samples, were selected for study and identification. Eight genera of the perfect yeasts were represented in the 149 cultures of this group. These were *Saccharomyces*, *Zygosaccharomyces*, *Hanseniospora*, *Debaryomyces*, *Pichia*, *Hansenula*, *Zygopichia* and *Torulaspora*. In the 82 cultures of the imperfect yeasts, the 7 genera represented were *Torulopsis*, *Mycoderma* as defined by Leberle and by Lodder, *Kloeckera*, *Rhodotorula*, *Asporomyces*, *Schizoblastosporion* and *Mycotorula*. The designation of *Mycotorula* is made with reservation, because of the present confused status of the taxonomy of the *Mycotoruloideae*. In addition to observations on the ecology of the cultures isolated, certain studies were made relative to the general problem of the taxonomy of the genera mentioned.

A26. *Yeasts Associated with the "Sugaring" of Dried Prunes and Figs.*

E. E. Baker and E. M. Mrak, University of California, Berkeley.

Dried prunes and figs commonly become covered with a white sugary substance during storage before processing and occasionally in cartons after processing. It is generally believed that this substance is composed of sugar crystals. Microscopic examinations of "sugared" dried-fruit samples obtained from all prune- and fig-producing sections in California have shown that this substance consists of a mixture of yeast cells and sugar crystals. This finding has been verified by the isolation of yeast cultures from practically all "sugared" samples.

The genera and sub-genera of the sporulating yeasts isolated were in the order of their frequency of occurrence: *Zygosaccharomyces*, *Hansenula*, *Saccharomyces*, *Debaryomyces* and *Zygopichia*. Several cultures of imperfect yeasts and a few cultures of bacteria were also isolated. When isolations were made from an enrichment medium containing 45° to 50° sugar, a great majority of the cultures obtained were species of *Zygosaccharomyces*. Isolations from a 15° Balling enrichment gave a greater distribution of genera, although the genus *Zygosaccharomyces* was still found more commonly than any other.

The cultures were inoculated into a medium containing yeast extract, primary potassium phosphate, and various amounts of invert sugar. Organisms capable of developing at invert-sugar concentrations up to and including 60 per cent were predominately *Zygosaccharomyces*; those developing up to and including 50 per cent were predominately *Hansenula* and those developing up to and including 40 per cent were predominately *Saccharomyces* and imperfect types. Only one culture of imperfect yeast failed to develop in the medium containing 30 per cent or more of invert sugar. No obligate osmophilic yeasts were encountered.

A27. Observations on Certain Film-Forming Yeasts. W. V. CRUESS AND L. HOHL, University of California, Berkeley.

Several strains of film-forming yeasts were isolated from Spanish Sherry and from mixed cultures of Spanish Sherry yeasts obtained for experimentation on application of the Spanish "flor" procedure in Sherry making. Several other strains were isolated from mixed cultures obtained from France. The general morphology, action on various carbohydrates, spore formation, alcohol production, alcohol tolerance and various other characteristics of these yeasts were determined. The results may be stated as follows. The typical Spanish "flor" yeasts are strongly oxidative in action on the alcohol, fixed acid, and acetic acid of wine. They impart a characteristic bouquet and flavor similar to that of young wines of Spanish Manzanilla or Fino character. Alcohol tolerance for film formation is high for the fermentive types, from 14 to 16 per cent by volume, and low for the non-fermentive types, about 10 per cent. Tolerance of the fermentive types is high for sulfur dioxide; from 200 to 500 parts per million, depending on the medium.

A28. Some Lactobacilli Encountered in Abnormal Musts. REESE VAUGHN AND HOWARD C. DOUGLAS, University of California, Berkeley.

One hundred and thirteen strains of bacteria of the genus *Lactobacillus* were isolated from samples of muscat grape musts (*Vitis vinifera var. Muscat of Alexandria*) which had spoiled while undergoing natural fermentation. These abnormal musts were high in volatile- and fixed-acid content and possessed an off-flavor, known as "mousiness". The activity of the yeasts had ceased because of the combined effects of high fermentation temperatures and large quantities of volatile acid. Previous investigations (Vaughn) had shown that the abnormal quantities of volatile acid noted in these samples resulted from rapid acetification brought about by the associative action of yeasts (*Saccharomycetaceae*) and *Acetobacter* organisms. The lactobacilli isolated, while not producing significant quantities of volatile acid, were important because of the ability to form large amounts of fixed acid and a disagreeable off-flavor.

The peculiar "mousey" off-flavor was produced by all the strains isolated, when they were grown in grape-must or grape-concentrate media. This off-flavor has been noted by several investigators as a characteristic of certain species of *Lactobacillus*. Under the conditions of our experiments many authentic strains of *Lactobacillus* produced this off-flavor when grown in grape-musts.

Former studies on the lactobacilli causing spoilage of musts and wines have stressed the importance of the gas-forming types of *Lactobacillus*. In this investigation only non-gas-producing strains were isolated. The majority of these cultures appears to be closely related to *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.*

A29. The Fermentation of Unripe Cigar-Leaf Tobacco. D. W. McKinstry, D. E. Haley and J. J. Reid. Pennsylvania Agricultural Experiment Station, State College.

In order to escape loss from disease or frost, growers frequently harvest cigar-leaf tobacco in an immature state. Such tobacco does not undergo a satisfactory fermentation and usually is lost by spoilage during the aging process.

Investigation has shown that the fermentation of unripe cigar-leaf tobacco is characterized by the development of a Gram-negative flora rather than by the development of the characteristic Gram-positive flora of a satisfactory cigar-leaf fermentation. The number of Gram-negative rods developing upon immature tobacco has been found to exceed 1×10^9 per gm., as measured by plate counts on nutrient agar. All pure-culture isolations of the rods studied have been found to ferment the common carbohydrates, including lactose, with the formation of gas. Most of these organisms have proved to be pigment producers in nitrate-peptone broth, although the soluble green pigment has been seldom noted in other media.

Chemical investigations have revealed that the development of the Gram-negative flora upon immature cigar-leaf tobacco is associated with the following: higher content of soluble organic nitrogen in unripe than in ripe tobacco; lower content of malic and citric acids in unripe tobacco; and the presence in unripe tobacco of reducing sugars, a type of compound almost negligible in ripe cigar-leaf tobacco.

A30. *Certain Factors Influencing Nitrification and Nitrate Accumulation in Soils under White Pine Forests.* JOHN M. HALE AND W. V. HALVERSEN, University of Idaho, Moscow.

The reclamation of white pine forest soils for farming has presented interesting bacteriological problems which concern the decomposition of the forest litter and the adaptation of the soil flora to a condition that will supply plant food to successive forage and cereal crops. The present study deals with: (1) methods for increasing the rate of nitrification in these soils; (2) factors which cause an increase in the number of nitrifying bacteria; (3) factors which inhibit nitrification; and (4) factors which have a beneficial influence on the rate of nitrate accumulation. Many previous researches have failed to discriminate between the nitrifying capacity and the nitrate-accumulating power of the soil.

In the present study a relative count of the nitrifying bacteria in soil has been made. The number of nitrifying bacteria was assumed to be an index of the intensity of nitrification. The nitrate-accumulating power was found by determining the amount of nitrates which accumulated in soils under a wide variety of conditions and treatments. The study showed that relatively few nitrifying bacteria exist in forest soils, but that under laboratory conditions nitrates will accumulate in such soils. This process is slow, however, as compared with that of nitrate accumulation in soils treated with nitrogen-rich fertilizers. There are substances in the forest litter which have an inhibitory influence on nitrate accumulation. These can be overcome with proper fertilizer amendments. The number of nitrifying bacteria may be increased markedly by treatment with nitrogen-rich fertilizers.

A31. *Taxonomic Relationships of Certain Non-Spore-Forming Rods in Soil.* H. J. CONN, New York State Agricultural Experiment Station, Geneva.

In the soil there are numerous non-spore-forming bacteria having the following characteristics: short rods, sometimes coccoid, with a tendency to be pleomorphic; Gram-negative or weakly Gram-positive; flagella sometimes absent, sometimes one, sometimes few (2–5) peritrichic, but never as a polar tuft; producing no acid from sugar except carbonic. In addition to miscellaneous soil saprophytes like *Bacterium globiforme* and

Bacterium radiobacter, this group includes the legume nodule and crown-gall organisms, and probably other plant pathogens.

Investigations show that certain species from animal sources (milk, intestines, etc.), commonly grouped in the genus *Alcaligenes*, seem to have the same general characteristics. They should not be grouped separately from the soil forms just because of a difference in habitat. Possibly some of the soil forms should be put in the genus *Alcaligenes*; but the status of this genus depends on whether its type species, *Alcaligenes fecalis*, can be recognized. Most bacteriologists recognize, under this name, any fecal organism which turns litmus milk alkaline; there is some question, therefore, whether the species can be accurately identified. This point is under investigation at present.

If *Alcaligenes* is a valid genus, it is suggested that it be placed in a family with *Rhizobium*, *Chromobacterium*, and those species of *Phytomonas* having the characteristics outlined above. For this family the name *Rhizobiaceae* is proposed.

A32. Moisture Changes and Their Effect on Soil as a Bacterial Medium.

H. J. Conn, New York State Agricultural Experiment Station, Geneva.

Experiments have been conducted on sterilized soil inoculated with either *Bacterium radiobacter* or *Bacterium globiforme* after adding glucose, calcium carbonate, potassium sulfate, and ammonium phosphate. Microscopic counts of the bacteria were made 7 days after inoculation.

Five soil samples, representing 3 distinctly different soil types, were used. When the tests were made, the soils were adjusted to approximately 50 per cent of their moisture-holding capacity; but, previous to sterilization and inoculation, they had been held at various moisture levels, namely, air-dry, 25, 35 and 45 per cent, respectively, of the water-holding capacity of each soil.

The soils studied, if allowed to become air-dry, were impaired as media for the growth of the organisms, even after bringing up to the optimal moisture content and adding complete nutrients. It proved possible to restore air-dry soil to a normal condition in this respect by holding it at 35 per centof its water-holding capacity for about 2 weeks before adding the nutrients, sterilizing and inoculating.

Experiments were carried on to show whether the improvement of soil after moistening might be due to changes brought about in it by growth of the natural soil flora during the 2-week period while the soil was moist, or due to release of alkalis during this period that might possibly have been fixed by the soil colloids while in an air-dry condition. Indications were obtained that fixation of potassium might be one of the factors involved; but results varied too greatly to be conclusive.

A33. Variations in the Microflora of Wheat Roots Following Soil Amendments. CHARLES THOM, FRANCIS E. CLARK, M. L. FIERKE AND HURLEY FELLOWS, U. S. Department of Agriculture, Washington, D. C. and Manhattan, Kan.

To determine the effects of soil amendments upon the microflora of the rhizosphere, potted soils fertilized heavily (1) with chicken manure or (2) with chopped green alfalfa, together with unfertilized checks, were planted to wheat. The soil employed was naturally infested with the take-all fungus, *Ophiobolus*; the disease was completely suppressed in the fertilized soils. Plate counts of micropopulations in the alfalfa-treated and in the manured soil showed abrupt increases within from three to five days to maxima of six hundred million and fifteen hundred million, respectively, followed by subsequent decreases toward the count of from thirty to eighty million in the check soil. Contrasting types of microörganisms developing during the period of rapid decomposition persisted for several weeks.

A comparison of the microflora associated with the root surfaces themselves revealed high counts of from one to two billions upon roots both in untreated and in treated soils. The types of bacteria involved contrasted sharply. The majority of organisms isolated from wheat roots grown in a chicken-manured soil were Gram-positive corynebacteria, readily inhibited in culture by the addition of crystal-violet, capable of reducing nitrates, and generally requiring organic nitrogen for growth, whereas predominating types associated with roots in alfalfa-treated soils, although corynebacteria, did not reduce nitrates, but were more active in carbohydrate fermentation. Organisms isolated from roots in treated soils differed from those from roots grown in untreated soil. Manifestly, the type of amendment affects the character, if not the numbers, of microörganisms directly associated with the wheat root system.

A34. The Fermentation of Definite Nitrogenous Compounds by Members of the Genus Clostridium. H. A. BARKER, University of California, Berkeley.

The primary function of putrefactive spore-forming anaerobes in nature is the decomposition of nitrogenous organic compounds. The object of the present investigation has been to elucidate the specific chemical activities of different species in the process of putrefaction by finding out the particular nitrogenous compounds decomposed and the products formed. This has been done by the use of anaerobic enrichment cultures containing definite nitrogenous compounds (glutamic and uric acids) as the only sources of energy. Numerous strains were isolated from the enrichment cultures and their morphological and physiological characteristics were determined.

Glutamic acid is fermented to butyric and acetic acids, carbon dioxide, ammonia and hydrogen by organisms that are universally distributed in soil. The ability of pure cultures to ferment glutamic acid is readily determined with a semisolid-agar medium containing this substance and a little yeast extract. All glutamic acid-positive strains are physiologically similar and have been identified as *Clostridium cochlearum* or *Clostridium tetanomorphum.*

Uric acid is rapidly converted anaerobically into ammonia, carbon dioxide and acetic acid. The causative organism, *Clostridium acidiurici,* develops well only on media containing urates.

The available data indicate the specificity of different organisms in putrefactive processes, the usefulness of definite nitrogenous compounds in isolating particular *Clostridium* species and the taxonomic value of the ability to ferment such compounds.

A35. Foxing and Deterioration of Paper. T. D. Beckwith, W. H. Swanson and T. M. Iiams, University of California, Los Angeles, and Huntington Library and Art Gallery, San Marino, Calif.

The deterioration of paper may be induced by a variety of factors, but the present work concerns the relationship of fungi to the process. The effects of 55 fungi isolated from paper have been studied. Sizings and fillers may further fungous growth and iron definitely is a stimulant, either in the presence or absence of sizing. The color of foxing ordinarily is unrelated to chromogenesis of the fungus but results from the nature and tint of the by-products formed. Growth of these microorganisms brings about the production of compounds which may be extracted by a solution of weak base and which are similar to humous structures in certain respects. These fungi can destroy the sheet of tissue inclusive of α-cellulose. Their metabolic demands for water are low. An explanation for the characteristics of foxed paper is advanced therefore. Technics for the recognition of fungous vestiges in paper have been elaborated.

A36. Bactericidal Activity of "Royal Jelly" of the Honey Bee. C. S. McCleskey and R. M. Melampy, Louisiana State University and Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, University, La.

"Royal jelly", the secretion of the pharyngeal glands of the worker honeybee which is the food of the queen, has pronounced bacteriostatic and bactericidal properties. *Escherichia coli* and *Eberthella typhosa* are inhibited by a concentration of 1–50 of the natural jelly in nutrient broth; *Staphylococcus aureus* and *Bacillus metiens* by a concentration of 1–100. The germicidal activity is markedly influenced by temperature. When 1 cc. of broth culture was added to 1 cc. of a 1–5 dilution of "royal jelly" at 43° to 45°C., the organisms were destroyed in 15 seconds; at 23° to 25°C., in from 10 to 30 minutes; while at 5°C. the organisms survived for 2 days. The bactericidal potency was rapidly reduced by neutralization of the natural acidity of pH 4.4 to 4.6. Sterilization of test cultures required only a few minutes at pH 4.6 (25°C.), but required 2 days at pH 7.0. The bactericidal capacity was largely lost when "royal jelly" was filtered through paper; it was found in the sediment after centrifugation. The active principle was removed from "royal jelly" by extraction with ethyl alcohol and with acetone. From these extracts, crystals were obtained which seemed to possess the bactericidal principle.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

One Hundred and Thirty-first Meeting, Philadelphia County Medical Society Building, April 26, 1938, Philadelphia, Penna.

A Comparative Study of Media Employed in the Isolation of Typhoid Bacilli from Feces and Urines. *Cora B. Gunther and Louis Tuft*, Pennsylvania State Health Laboratories, 34th and Locust Sts., Philadelphia, Pa.

In this study, 2245 feces and urines were cultured for typhoid bacilli employing various media in a comparative way. Wilson-Blair's bismuth-sulphite medium was shown to be superior to Endo, eosin-methylene-blue and Leifson's desoxycholate-citrate media. The Difco modification of Wilson-Blair medium, used in these tests, is superior to Wilson's original medium, being easier to prepare, relatively more stable and more sensitive. It is useful for isolating most of the important *Salmonellas* but not for the dysentery group. Leifson's selenite enrichment medium was shown to be superior to 30% glycerine enrichment. A technic was evolved in which a loopful of feces is streaked directly on each of two Wilson plates and an emulsion also made in selenite F. After incubation, a loop of emulsion is streaked on Wilson medium. It was found that 25.5% of the total positives were positive on Wilson, direct streak, and negative after enrichment and 17 per cent were positive after enrichment and negative by direct streak. Both methods of culturing are recommended since some are missed by each technic. A change in routine technic from 30% glycerine enrichment and streaking on Endo to the technic described above has increased the positive typhoid isolations almost 50%.

The Chemistry of a Nucleoprotein Antigen —"Labile Antigen"— from Hemolytic Streptococci of Lancefield Group A. *M. G. Sevag, D. Lackman and J. Smolens*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

An antigen designated "labile antigen" and believed to be identical with Griffith's type-specific agglutinogen of hemolytic streptococci, Lancefield's Group A, has been described by Mudd, Czarnetzky, Lackman and Pettit (Jour. Immunol., **34**, 117, 1938). This antigen was first obtained from the aqueous extracts of streptococci disintegrated by audible sound treatment by Chambers and Flosdorf (Proc. Soc. Exper. Biol. and Med., **34**, 631, 1936). Chemical studies show that this antigen is a nucleoprotein and analyzes 2.65% phosphorus, 15.79% total nitrogen, 9–13% reducing sugar calculated as glucose and less than 2% ash. On treating with 0.5% sodium carbonate solution at 55°C for 1–2 hours, this substance is split into a protein and nucleic acid.

The protein component, on further treatment with N/20 NaOH overnight in the refrigerator and for one hour at 55°C, is rendered free of the residual nucleic acid. The analysis of this protein shows 14% total nitrogen, negative purine nitrogen, pentose and phosphorus. It shows 5.8% reducing property calculated as glucose after acid hydrolysis. Serologically, it is active with sera prepared against Lancefield's Group A and inactive against streptococcus Groups B and F. It produces immune sera upon injection into rabbits.

The nucleic acid fraction is treated with chloroform (Sevag, Bioch. Zt., **273**, 419, 1934) to remove the protein. On analysis it shows 9.25–9.67% phosphorus, 15.79–15.90% total nitrogen, 10.24% purine nitrogen, positive pentose reaction and 14.4–14.81% reducing sugar calculated as glucose.

These nucleic acids are in certain instances serologically active, depending upon the method of isolation; precipitation with mineral acids seems to destroy this property. On the other hand, precipitation with weakly acidulated alcohol seems to preserve their activity.

SOME REACTIONS OF SULFANILAMIDE WITH NUCLEOPROTEINS AND A SUGGESTED MECHANISM OF THE ACTION OF SULFANILAMIDE. *E. J. Czarnetzky* and *H. E. Calkins*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Hope of finding a chemical entity capable of destroying bacteria specifically without injury to the host seems to have been realized in the discovery of the action of sulphanilamide on streptococci. In order to take advantage of this finding in a search for new specifics which would combat other types of bacterial infections, it is necessary to determine the basic chemical reactions underlying the bacterial action of sulphanilamide.

There is good evidence for the belief that the surface of β-hemolytic streptococci of Lancefield Group A contains an antigen which is partly composed of nucleic acid. This antigen can be isolated in a purified form, and when treated with sodium bisulphite or sulphanilamide it becomes lytic for red blood cells *in vitro*. Yeast nucleic acid and vitamin B_1 are made hemolytically active in the same manner by both reagents. It is known that the reaction between vitamin B_1 and sodium bisulphite results in the formation of a sulphonic acid derivative of the pyramidine ring of the vitamin. Such a sulphonic acid derivative is hemolytic. A similar chemical reaction takes place between sulphanilamide and yeast nucleic acid or streptococcal nucleic acid, resulting in the production of hemolysins, which are compounds of sulphanilamide and various nucleosides.

A consideration of the toxic effects of sulphanilamide, which include hypochromic anemia and granulopenia, seems to be in harmony with the fact that sulphanilamide may react with nucleic acid compounds with a resultant formation of hemolysins.

ONE HUNDRED AND THIRTY-SECOND MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, MAY 24, 1938, PHILADELPHIA, PENNA.

MOLECULAR WEIGHT, ELECTROCHEMICAL AND BIOLOGICAL PROPERTIES OF TUBERCULIN PROTEIN AND POLYSACCHARIDE MOLECULES. *Florence B. Seibert*, *Kai O. Pedersen* and *Arne Tiselius*, Institute of Physical Chemistry, University of Uppsala, Uppsala, Sweden and the Henry Phipps Institute, University of Pennsylvania, Philadelphia, Penna.

Molecular size and homogeneity of the protein molecules in tuberculin which elicit the tuberculin skin reaction were determined by means of sedimentation in the Svedberg ultracentrifuge, diffusion by the method of Lamm and electrophoresis in the Tiselium apparatus. Chemical analyses for nitrogen, polysaccharide and nucleic acid, and biological tests for potency, toxicity and precipitin reaction were made.

Six homogeneous molecules were isolated, one of about 10,000 molecular weight from bovine and one of about 17,000 molecular weight from timothy-bacillus culture filtrates. The polysaccharide had a molecular weight of about 9,000 and differed remarkably from all polysaccharides hitherto studied in exhibiting molecular homogeneity.

A homogeneous protein with molecular weight of about 32,000, isoelectric point pH 4.3 and isoionic point pH 4.7, was isolated from human type filtrate. It gave a high precipitin titer, was very toxic and produced the anaphylactic type of local skin reaction in tuberculous guinea-pigs.

PP D was separated into a mildly potent fraction with molecular weight about 9,000, an heterogeneous but highly potent fraction, and an homogeneous fraction with molecular weight of about 16,000, which was non-antigenic and found to be a stretched molecule by means of double refraction in flow.

STUDIES ON TETANUS TOXOID. *Herman Gold*, Sharp & Dohme Laboratories, Glenolden, Pa.

The injection of tetanus toxoid produces in most individuals a slight to moderate local reaction lasting 1 to 3 days. The alum-precipitated toxoid produces a subcutaneous nodule, usually absorbed in 2 to 4 weeks.

In agreement with Jones it was found that the antitoxin content was higher in individuals receiving two doses of alum-precipitated toxoid than in those receiving 3 doses of plain toxoid or plain toxoid plus 0.4% alum.

Loss of antitoxin in all persons, regardless of the kind of toxoid injected, was most marked the first few months following the initial immunization course. By the end of a year the majority of the immunized individuals showed less than 0.1 unit of antitoxin per cc. of serum—the minimum protective amount. The first dose of alum-precipitated toxoid seemed to act as "sensitizer"; following the second dose there was a prompt antitoxin increase in the blood.

The immunity response of different individuals following the initial dose series of alum toxoid is variable, making it necessary to administer a "repeat" dose of toxoid whenever an injury occurs. In most cases, five days after injection of the "repeat" dose the antitoxin titer was up to or above the protective level. Following the "repeat" dose of toxoid the protective titer was maintained for a longer period of time than after the initial course of immunization.

INTRANASAL DIPHTHERIA IMMUNIZATION IN HUMANS. *LeRoy J. Wenger*, *Bettylee Hampil* and *Peter Masucci*, Mulford Biological Laboratories, Sharp & Dohme, Glenolden, Pa.

Today's problem in mass immunization against diphtheria is maintaining the resistance produced by immunization. Dr. Jensen suggested (Proc. Royal Soc. Med., 30: 1117–48, 1937) that a single dose of aluminum-hydroxide precipitated toxoid be given subcutaneously; and that the immunity produced be reinforced and prolonged by the intranasal instillation of plain toxoid four weeks after the injection.

Diphtheria-antitoxin response to single doses of alum-precipitated toxoid and of aluminum-hydroxide precipitated toxoid in same human age group were compared. A concentrated diphtheria toxoid for intranasal instillation was developed at the Mulford Biological Laboratories. Intranasal instillation in normal guinea-pigs showed, 4 weeks after 3 series of treatments, 0.5 unit of antitoxin per cc. of blood. Intranasal instillation of concentrated toxoid in guinea-pigs, 14 weeks after subcutaneous injection of a human-dose diphtheria toxoid, precipitated, showed 5 days after the last instillation more than 6 units of antitoxin per cc. Twelve young adults, still Schick positive 3 months after subcutaneous injection of a dose of alum-precipitated toxoid, were given 3 weekly intranasal instillations of concentrated diphtheria toxoid. One week later in 10 of the 12, there was an increase in antitoxin titer of from 10 to 250 times the titer before the intranasal instillation.

CONNECTICUT VALLEY BRANCH

SPRING MEETING, MAY 14, 1938, WESLEYAN UNIVERSITY

SOME FACTORS INVOLVED IN TESTING DISINFECTANTS. *Elizabeth F. Genung*, Associate Professor of Bacteriology, Smith College, Northampton, Mass.

A comparison of seven different methods of testing disinfectants, described in the literature, revealed startling discrepancies in the results obtained when identical organisms were employed by the same worker throughout. Some methods were outstanding in uniformity of results, simplicity and efficiency. It was clearly demonstrated that a chemical should not be evaluated on the basis of one test alone.

A modification of a method used for studying bacteriophage, "'phage method," was found to adapt itself to the testing of chemicals and is suggested as a preliminary test for the potential value of a disinfectant. At least 15 cc. of nutrient agar are poured into a sterile Petri plate, allowed to harden, and 0.2 cc. of the culture is spread, evenly, over the surface with a sterile bent wire or glass rod. When dry, one drop of the disinfectant is placed on the surface and allowed to absorb. The plates are then incubated under porous tops for twenty-four hours. A well defined clear area indicates by its size, freedom from colonies, etc. the bactericidal or inhibitory properties of the chemical. Blood, serum or other organic material may be added to the agar.

The results should be checked by, at least, one other method. The cover-slip method, described by Jensen and Jensen (1933) is found to give uniform results as well as being more convenient to operate than the loop method, usually recommended, especially when very short time intervals are employed.

FACTORS GOVERNING THE MORPHOLOGY OF MONILIA ALBICANS. *Lynferd J. Wickerhan*, Department of Bacteriology, Yale University.

A large number of strains of *Monilia albicans* obtained from various sources were studied under various environmental conditions in slide cultures.

Strains were classified into five types on the basis of the comparative number of blastospores and chlamydospores produced on the hyphae in cornmeal agar preparations. Type I cultures produced practically all chlamydospores, type V cultures produced only blastospores. These type characteristics were hereditary, but could be modified by growing the organisms on other media. On a richly nutritive medium such as glucose agar the vegetative rather than the sporulative phase was promoted, and resulted in the production of large numbers of blastospores even by strains which on cornmeal agar formed chlamydospores exclusively.

Chlamydospores were not formed in any medium at a temperature of 30°C. or higher.

EPIDEMIOLOGIC SIGNIFICANCE OF SERO-TYPING IN CHICK PARATYPHOID. *Carl F. Clancy and Erwin Jungherr.* Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Conn.

Three outbreaks of paratyphoid in chicks were observed in poultry stock on premises with a previous history of paratyphoid infection. The organisms obtained from the original and second outbreak in the respective cases were subjected to biochemical and serologic identification, the latter on the basis of antigenic structure according to the Kauffmann-White scheme.

In the first case *Salmonella typhimurium* was recognized as the causal agent and reisolated 1 month following the original outbreak. In the second case *Salmonella montevideo* was involved and recovered in specimens submitted 14 months later. *Salmonella oranienburg* was isolated in the third case and found again 25 months later. The occurrence in chicks of rare sero-types which have hitherto been regarded as essentially human pathogens suggests lack of species-specificity in the genus *Salmonella.* On the other hand, the recurrence of the same sero-type in repeat-outbreaks at intervals of 1 to 25 months points towards a more or less endemic occurrence of certain types, for the recognition of which sero-typing appears to be of special value.

THE GROUPING OF HEMOLYTIC STREPTOCOCCI ASSOCIATED WITH DISEASE IN CHILDHOOD. *Paul L. Boisvert*, Department of Pediatrics, Yale School of Medicine.

Lancefield has demonstrated that hemolytic streptococci can be divided into distinct groups by a precipitin test. Although human pathogenic strains usually fall in her serological group A, other groups have been found responsible for human disease.

We were anxious to obtain some idea of the frequency in human beings of infection by these other groups, especially in infants and children, in whom resistance to the hemolytic streptococcus is relatively low.

During a period of one and one-half years, all available strains of hemolytic streptococci from 248 patients, with streptococcal disease, were grouped by the Lancefield method. 217 of these patients were infants and children.

All of the 408 strains from these patients fell in group A. Hemolytic streptococci belonging in groups B, C, and G have occasionally been encountered, but there was no evidence that they were causing disease.

The findings suggest that infants and children are no more susceptible than adults to hemolytic streptococci of groups other than A. In fact, the precipitin test gave remarkably consistent results both in these young individuals, and in the adults who were studied.

A Comparison of Properties of Toxic Extracts of the Eberthella-Salmonella Group. *Eleanor Cornell Bower*. Smith College, Northampton, Mass.

Toxic extracts have been prepared from *Eberthella typhosa*, *Salmonella enteriditis*, *Salmonella aertrycke*, *Salmonella schottmulleri*, and *Salmonella suipestifer* by holding washed suspensions of 18-hour beef-heart infusion agar cultures at 58–60°C. for 4 hours, centrifuging, and filtering the supernatant through Berkefeld N or W candles. These filtrates, when injected intraperitoneally, would kill 3 out of 5 twenty-gram mice in doses ranging from 0.3 to 0.8 ml., depending on the organism used in preparing the extract. All extracts suffered some diminution in their lethal potency after heating at 100°C. for 1 hour, although the same amount of extract which, unheated, would kill 3 out of 5 mice in 24 hours, still retained the ability to make the injected animals ill.

The extracts had the same physiological effect on injected mice, differences observed being quantitative rather than qualitative. At autopsy, the most striking and typical finding was an involvement of the small intestine which was empty, transparent in the duodenal and jejunal regions, and filled with a yellowish fluid.

Specific agglutinins for both O and H antigens were obtained by injecting rabbits with small graded doses of the toxic extracts. The antisera did not possess any very potent antitoxic immunity in so far as this property could be demonstrated by passive protection experiments with mice. Antisera injected in 0.5 ml. amounts one-half hour prior to the administration of 2 minimum lethal doses of extract seemed to increase the average time to death, but the animals were always ill and the percentage of survivors never very high. Homologous antisera gave the best protection. Heterologous antisera to extracts of organisms containing common O fractions, while less protective than homologous sera, seemed to have more antitoxic potency than antisera to extracts of organisms not sharing antigens in the soma.

Variation Studies of Mycobacteria: Colonial and Cellular Morphology. *Harriette D. Vera*. Department of Bacteriology, Yale University.

Four human and two bovine strains of the tubercle bacillus, one strain of *Mycobacterium smegmatis* and several strains of soil mycobacteria were grown under various conditions for different periods of time. They were cultured on Long's agar medium and on meat-extract agar. They were grown in atmospheres containing varying proportions of oxygen, nitrogen, and carbon dioxide. Careful records, including photographs, were made of the colonial characteristics.

Growth and pigmentation of colonies were increased by an increased oxygen supply. When the oxygen supply was restricted, the colonies were white or colorless, and they tended to be somewhat more smooth than those grown in the presence of air.

Approximately 300 colonies were

embedded in paraffin and were sectioned vertically, 2-4 micra thick. Sections of all colonies were stained by the Ziehl-Neelsen technique; sections of most of the colonies were also stained by the Gram method. No section of any colony was seen that was composed exclusively of acid-fast cells. The proportion of acid-fast cells, as well as their number, appeared to be increased by a high-oxygen atmosphere. Almost all colonies showed stratification in the dye reactions and in the cellular arrangement; the stratification pattern, however, was variable. The positions of the Gram-positive substance and the acid-fast cells corresponded closely, but were not identical. Very short coccoid forms occurred in colonies grown on meat-extract agar.

THE APPARENT OXIDATION-REDUCTION POTENTIALS OF BRIGHT PLATINUM ELECTRODES IN SYNTHETIC MEDIA CULTURES OF BACTERIA

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Received for publication November 19, 1937

The study of oxidation-reduction potential changes in culture of various organisms has been pursued by many investigators since Potter (1911) first noted that the electrode potential of an inoculated medium was more negative than a sterile control. The extensive literature which has ensued contains results of determinations obtained almost entirely by the use of complex media of unknown composition. A few workers (Elema, *et al.*, 1934, Allyn and Baldwin, 1932, Thorne and Walker, 1936) have used media of known chemical composition as a substrate for the growth of soil and related organisms in redox studies. Thus far, however, to the writer's knowledge no attempt has been made to make similar studies with pathogenic or potentially pathogenic bacteria. As a result of the recent work on growth accessory substances or vitamin requirements (Koser, *et al.*, 1935, 1936; Sahyun, *et al.*, 1936; Knight, 1937 and Mueller, 1937a, b) it is possible to make potential measurements of growing synthetic-medium cultures of these latter forms.

The potentiometric method has been found superior to the colorimetric method for measuring potentials of this type. Preliminary determinations made with a conventional type K potentiometer bridge have shown such an arrangement to be entirely unsatisfactory for measurements in nutrient broth and synthetic media. The range of values obtained by consecutive readings was too wide to allow significance to be attached to them. This undoubtedly was due, in part, to polarization phenomena. A vacuum tube null-point instrument was constructed which eliminated polarization effects and gave identical consecutive readings in poorly poised systems. Despite this, however, discrepancies between duplicate electrodes and identical culture vessels were still encountered in both nutrient broth and synthetic media cultures. These irregularities appeared to be of sufficient magnitude and importance to warrant further study. From theoretical considerations there were several possible factors that might be involved: (1) Since a certain amount of stratification is known to occur in the growth of organisms in liquid media it seemed of value to investigate the effect of agitation of the medium. (2) In view of somewhat conflicting statements in the literature regarding electrodes, several types were used. (3) The poorly poised condition of simple culture media solutions suggested that appropriate poising substances might be tested. (4) The control of oxygen tension is obviously of importance both because of interference with hydrogen transfer and because of an oxygen electrode effect. In the present paper are reported comparative data obtained in a study of the relation of these factors to the apparent oxidation-reduction potentials produced in broth and synthetic media. Although it is recognized that blank platinum electrodes may in the presence of hydrogen give a hydrogen electrode effect, this aspect of the problem was not investigated directly since the test organism, *Escherichia coli*, ordinarily liberates a certain amount of this gas in its growth processes.

METHODS

The vacuum tube circuit[1] is shown in figure 1. The vacuum tube heater voltage is set to approximately four volts by means of the 20-ohm variable resistance. Adjustment is made in the plate current line by manipulation of the potentiometers inserted therein so that no deflection of the galvanometer occurs when the tap key *A* is closed. Approximately 0.1 milliampere is read on *M*. During this period of primary balance the control grid is isolated and floating and as a result acquires a potential value at which the grid current is practically eliminated. The maximum grid current at any time was 10^{-11} ampere. Finally this grid is biased to this latter value by closing the special air-insulated switch *B*, closing *C* and adjusting the 6000-ohm and 600-ohm potentiometer resistances. The point of balance is again detected by using the key *A*. Switch *B* is now opened and the unknown half cell is connected to one pole of *X* and the saturated calomel fiducial cell to the other pole. The former cell is connected to the latter by means of an agar potassium-chloride bridge using a saturated potassium-chloride liquid junction. The potential of the complete cell is then obtained as a direct reading on the potentiometer when *G* again shows no deflection. The calomel cell was prepared from electrolytic calomel, recrystallized potassium chloride and redistilled mercury. On checking against various buffers it was found to have the value 0.2364 volt at 37.5°C. A Leeds and Northrup lamp and scale type galvanometer having a current sensitivity of 0.025 microampere was used in most of the experiments. The performance of this instrument was checked against the more sensitive reflecting type having a sensitivity of 0.0005 microampere. The results were identical in all cases.

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[1] This circuit is a slight modification of one suggested by Dr. Otto H. A. Schmitt of Washington University, St. Louis.

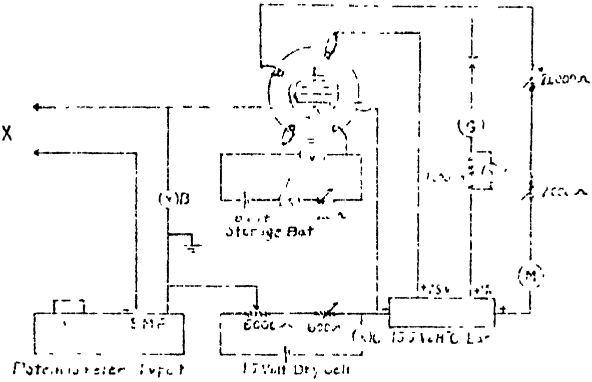


FIG. 1. VACUUM TUBE CIRCUIT

M, 0 1 milliammeter; *V*, 0 6 voltmeter; *G*, galvanometer; *X*, unknown half cell and reference half cell; *B*, air insulated switch; *A*, *C*, and *E* are simple toggle switches. Type 38 R. C. A. tube.

The electrodes were connected with the measuring apparatus through small mercury-containing steel cups embedded in high-melting-point paraffin. This material offered a suitable means of insulation because of its high resistance. Long, rectangular blocks of paraffin containing these cups were supported by the upper edges of the constant temperature bath in which the culture cells were incubated at 37.5 ±0.02°C. The culture vessels were 180 ml. electrolytic beakers without pours. These were closed with alkali-treated rubber stoppers containing five holes, two for electrodes, one for the agar potassium chloride bridge, one to allow for filling with medium, and a central one for the stirring arrangement. The latter was sealed either with mercury or oil. Mercury appeared to be more efficient and was easier to clean out than the oil; so it was used in the majority of the agitation experiments.

Electrodes were prepared by sealing into soft glass tubing of the desired size. Before the seal was made, a piece of 28-gauge platinum wire was silver-soldered to the part of the electrode entering the tube so that this small gauge wire could be brought out the other end and the glass sealed about it. This gave a glass tube sealed at both ends carrying the contact wire to which, on the outer end, a small piece of copper wire was silver-soldered for making contact with a mercury cup. An electrode of this type was easy to clean and handle.

For cleaning the electrodes, hot chromic acid, nitric acid and *aqua regia* were tested. As far as could be detected no one method was superior. The nitric acid method has been used in practice because the action of *aqua regia* is somewhat vigorous on fine gauze or foil electrodes. Those cleaned by heating gave no better results than were secured by the other methods.

The stirring mechanism, of the mechanical type, was operated by a ratio motor with output of 86 r.p.m. From this, by a series of pulleys and belts, the stirrers were operated in from one to ten cells simultaneously at a speed of approximately 130 r.p.m.

All synthetic media were prepared by weighing the requisite C.P. or Reagent chemicals on an analytical balance and adding to sterile re-distilled water. The water-clear solution was then filtered through a Seitz pad or Berkefeld N candle and incubated to test for sterility. The basic synthetic medium, a slight modification of that of Koser and Saunders (1935), was composed of Na_2HPO_4 1.4 grams, KH_2PO_4 1.0 gram, NaCl 2.0 grams, $MgSO_4$ (anhy.) 0.1 gram, l-asparagine 3.0 grams, l-tryptophane 0.2 gram, d-glucose 2.0 grams and re-distilled water 1 liter. The final pH was 6.9. Standard sodium hydroxide was used to adjust the pH when additions such as cysteine hydrochloride were made. The nutrient broth used was composed of beef extract (Swift) 3.0 grams, peptone (Difco) 10.0 grams, NaCl 5.0 grams and re-distilled water 500 ml. This was adjusted to a pH of 6.9, sterilized in the autoclave, and to it added 500 ml. of sterile phosphate buffer (pH 6.9 at 37.5°C.). The buffer was present in a final concentration of M/30.

The culture vessels containing only the electrodes were sterilized by autoclaving. Before the electrodes dried, the medium, in 100 ml. quantities, was pipetted into the vessels. The agar potassium-chloride bridges were filled aseptically, inserted into the bung, and held in place by a section of close-fitting rubber tubing.

In the anaerobic experiments, commercial water-pumped cylinder nitrogen was purified by bubbling through sodium hydroxide solution and concentrated sulfuric acid, then run through a heavy wall pyrex combustion tube held in an electric furnace. The combustion tube was packed with small-gauge copper oxide wire and the latter was reduced in a stream of hydrogen before each experiment. Precautions were taken to blow all hydrogen out of the combustion tube before the culture vessels were connected. From the combustion tube the purified gas was led through a trap and then into a Y tube extending across and above the two rows of culture vessels. The nitrogen was bubbled through the medium in each vessel at approximately an equal rate. To accomplish this a piece of small-bore glass tubing was run through the center of each rubber bung and connected, using heavy-wall rubber tubing, to another piece of glass tubing of the same bore which projected at right angles from the nitrogen line. The two ends of glass tubing were approximated so that the chance of oxygen diffusing through the connection was lessened. Both pieces of tubing were stoppered with a small piece of non-absorbent cotton. It is realized that these conditions do not yield absolute anaerobiosis, but it was felt that they were sufficient for the desired studies.

In every experiment all vessels were checked microscopically and culturally for contamination at the end of each determination. Counts were also made at the conclusion of an experiment to determine the total number of bacteria per ml. The vessels were labeled alphabetically in order to prevent confusion when taking measurements.

A carefully checked stock strain of *Escherichia coli* which did not ferment sucrose was used in all determinations. Inoculations were made directly into the medium from a twenty-four-hour agar slant culture. One hundred ml. aliquots were then pipetted out and, thus, each inoculated culture vessel had practically an equal number of organisms. However, no further attempt was made to standardize the number of inoculated organisms for it was thought that if good agreement was obtained consistently in individual experiments, the duplication of experimental results should not be difficult.

Every electrode was examined for flaws at the conclusion of each experiment and all were checked against one another by measurement of dilute solutions of ferrous-ferric salt mixtures. If defects were noted the results for the corresponding vessel were discarded from the data.

All experiments were performed at least three times. In each determination an average of ten separate culture vessels were used, each of which contained duplicate electrodes.

The hydrogen ion concentrations of the various media were determined before and at the end of the incubation period by means of a glass electrode.

EXPERIMENTAL RESULTS

Stationary synthetic medium cultures

Under conditions wherein no agitation of the substrate occurred, the experimental results were at variance between vessels containing identical electrodes, medium, and number of inoculated organisms. In figure 2 typical time-potential curves obtained by using platinum wire spiral electrodes are shown. The double-line curve appearing under each letter represents a plotting of values for individual identical duplicate electrodes in the same culture vessel. Representatives were chosen from neither extreme, being selected from a group of ten vessels all of which were incubated at the same time. Other identical experiments always gave similar variations. The maximum difference shown, which is approximately 444 millivolts, exists between values obtained in separate vessels while the maximum between duplicate electrodes in any one vessel is 95 millivolts. In the majority of cases, readings became more nearly concurrent after the culture had been incubated thirty-six hours. There was no indication, when growth comparisons were made, that these or subsequent irregularities were due to differential growth in various vessels.

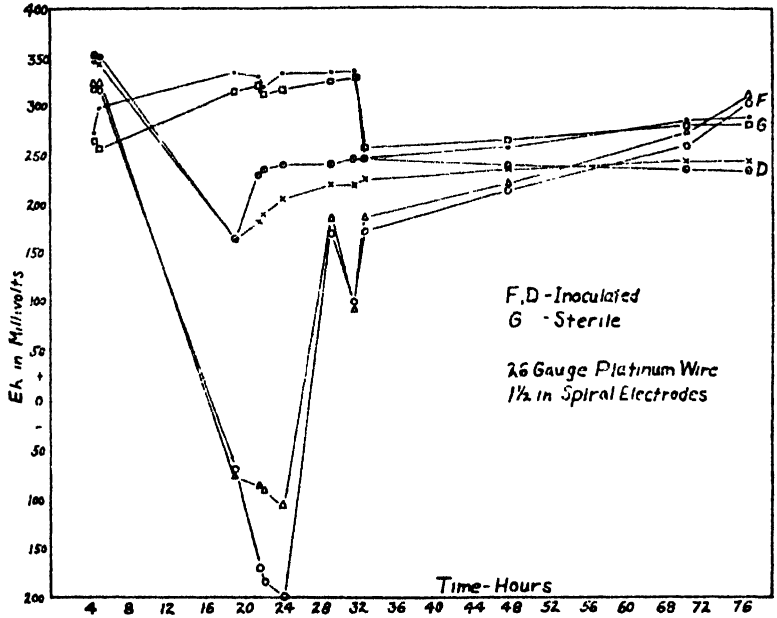


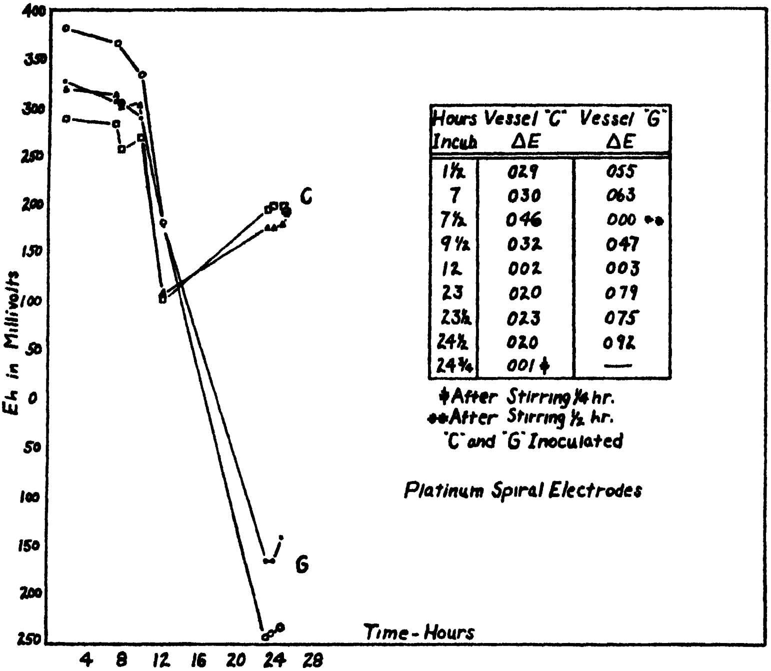
FIG. 2. TIME-POTENTIAL CURVES OBTAINED IN THE BASIC SYNTHETIC MEDIUM UNDER STATIONARY CONDITIONS

Each curve represents values for a single electrode

Results obtained by using other forms of platinum electrodes showed a shift in the general oxidation-reduction intensity which will be discussed later, but discrepancies were still apparent.

Intermittently agitated synthetic medium cultures

Since the irregularities shown in the previous experiment are not readily explicable on the basis of mechanical defects or methods used in cleaning the electrodes, the possibility is to be considered that localized growth, collection of gas bubbles, or temporary poisoning of one electrode might account for some questionable potential readings.



Hours Incub	Vessel "C" ΔE	Vessel "G" ΔE
1½	029	055
7	030	063
7½	046	000 ••
9½	032	047
12	002	003
23	020	079
23½	023	075
24½	020	092
24¾	001 ♦	—

♦After Stirring ¼ hr.
••After Stirring ½ hr.
"C" and "G" Inoculated

Platinum Spiral Electrodes

FIG. 3. EFFECT OF AGITATION ON TIME-POTENTIAL VALUES OBTAINED IN THE BASIC SYNTHETIC MEDIUM

ΔE, in millivolts, is equal to the difference between duplicate electrodes in the same vessel. Each curve represents values for a single electrode.

The plotted values in figure 3 are typical for cultures stirred for several short intervals during incubation. ΔE, in millivolts, is equal to the difference between duplicate electrodes in the same culture vessel at any one time. After seven hours' incubation under stationary conditions the electrodes, of the platinum wire spiral type, in vessel *G* gave a ΔE value of 63 millivolts. As a result of agitating for one-half hour ΔE was equal to zero. Likewise in vessel *C* the ΔE value was changed from twenty millivolts at twenty-four and one-half hours to one millivolt after stirring for fifteen minutes.

Better agreement, not necessarily absolute, was obtained between like duplicate electrodes in the same vessel under conditions of agitation, although there still remained a considerable difference among individual vessels containing the same medium. This again could not be accounted for by differences in growth or electrode defects.

Platinum electrode types in synthetic medium cultures under conditions of agitation

During the course of these experiments, various types of electrodes—platinum wire pin-point and spiral forms—as well as platinum foil and gauze types were tested. The time-potential relationships obtained in the basic synthetic medium plus 0.10 gram of cysteine hydrochloride are presented in figure 4. The curves in this figure were plotted from averages derived from duplicate electrodes in each vessel. In many cases ΔE values were rather large, so the mean is of questionable significance. These ΔE values ranged from 100 millivolts to zero millivolt, with approximately the same degree of irregularity appearing in both sterile and inoculated vessels.

The high values given by foil electrodes in the sterile medium, as shown by curves *E* and *D*, are typical for these and for gauze types. The potential in the same sterile medium was approximately 200 millivolts higher when determined by means of foil electrodes in contrast with readings with wire electrodes. The regularity of *E* and *D* as shown in the graph was not always as pronounced. In the inoculated medium, the wire electrodes and foil electrodes did not give the marked differences that were obtained in the sterile medium.

There was no agitation of the cultures between the twelfth hour and the twenty-ninth hour. This results in the appearance of a trough in the curve at this time. In other experiments where cultures were not agitated or were constantly agitated throughout the entire period, the greatest reducing intensity appeared earlier. The inoculated vessels, again, regardless of the electrode type, showed an approaching similarity of values after thirty-six hours' incubation.

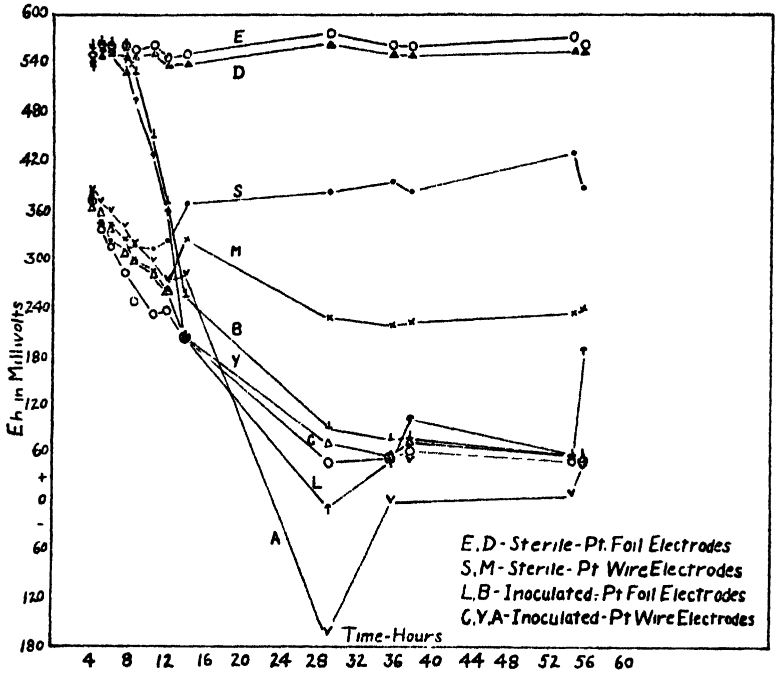


FIG. 4. TIME-POTENTIAL RELATIONSHIPS OBTAINED IN SYNTHETIC MEDIUM BY PLATINUM FOIL AND WIRE SPIRAL ELECTRODE TYPES UNDER CONDITIONS OF AGITATION

Each curve represents an average of the readings obtained from duplicate electrodes in the same vessel.

Since the presence of cysteine in the medium might be considered to be a cause of the irregular values, the experiment was repeated using the basic synthetic medium without cysteine. Similar results were obtained. In the experiment shown in figure 4 and in additional experiments of a similar nature, there was no case in which any one electrode type appeared to give more consistent results than the other types.

Nutrient broth cultures compared with synthetic medium cultures under conditions of agitation

Previous workers, using broth and more complex media of unknown composition, have reported close agreement between duplicate electrode and duplicate vessel readings. The nutrient broth medium was used in these experiments in order to compare the results obtained in it with those in a synthetic medium.

The data obtained by using broth medium were consistently in better agreement. The majority of the ΔE values were in a range of 0 to 5 millivolts, with an occasional higher value of 20 millivolts. The difference between culture vessels was of the order of 5 to 10 millivolts. At times, apparently when metabolic changes were quite rapid, this difference was in a few instances somewhat greater. The results here, in contrast to those obtained in experiments using synthetic media, were fairly consistent throughout the entire period of incubation.

Semi-poised synthetic medium cultures

In the data thus far presented there is a suggestion that the irregularities still prevalent in a synthetic medium culture are due either to polarization of electrodes or to the absence of sufficient oxidizing-reducing material to give stable true values in the presence of oxygen. Polarization of the electrodes due to the measuring apparatus was ruled out because the irregular values were reproducible over a period of time and in different experiments. The electrodes in question, when checked against an inorganic oxidation-reduction system, always gave extremely regular results independent of type, shape or size of the metal surface. These discrepancies appeared, then, to be due to a culture medium poorly poised in the presence of air. Consequently, a reversible inorganic oxidation-reduction system was added to the synthetic medium in concentrations small enough so as not to alter the growth of the organisms perceptibly. Preliminary results, obtained by growing the test bacterium in the synthetic medium and adding to the vessels (after irregularities were noted), small concentrations of potassium ferricyanide, were encouraging.

In figure 5 are recorded results obtained by allowing the organism to grow in the synthetic medium containing a 0.00031 *M* concentration of $K_3Fe(CN)_6$. Growth was excellent, and the results were regular for the first ten hours of incubation. Such variations as did occur were of a small magnitude. In the sterile medium ΔE values were of the order of 2 millivolts, while in the inoculated medium the maximum variation was greater (20 millivolts). Experiments in which the concentration of $K_3Fe(CN)_6$ was increased gave almost perfect data. However, growth in such cases was retarded to a slight extent. It should

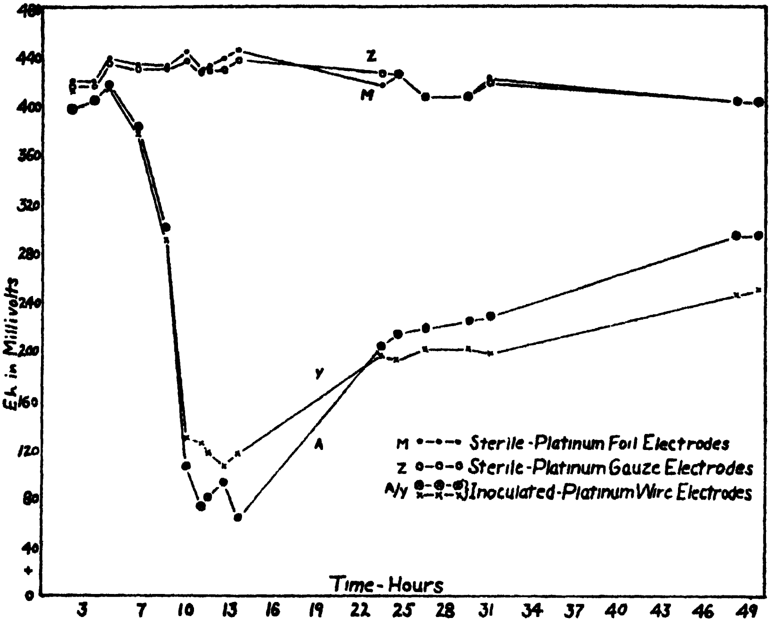


FIG. 5. TIME-POTENTIAL RELATIONSHIPS OBTAINED IN THE BASIC SYNTHETIC MEDIUM PLUS 0.00031 *M* CONCENTRATION OF $K_3Fe(CN)_6$ UNDER CONSTANT AGITATION

Each curve represents an average of the readings obtained from duplicate electrodes in the same vessel.

be noted that this poising of the medium dispelled differences in electrode types.

Synthetic medium anaerobic cultures

In view of certain peculiarities in the electrode effects previously noted, it seemed likely that some irregularities could be eliminated by removing atmospheric oxygen from the culture medium. The basic synthetic medium was chilled before being put into the culture vessels so that most of the oxygen could be removed by the stream of nitrogen before the temperature rose to a point where growth would become vigorous. One hour was

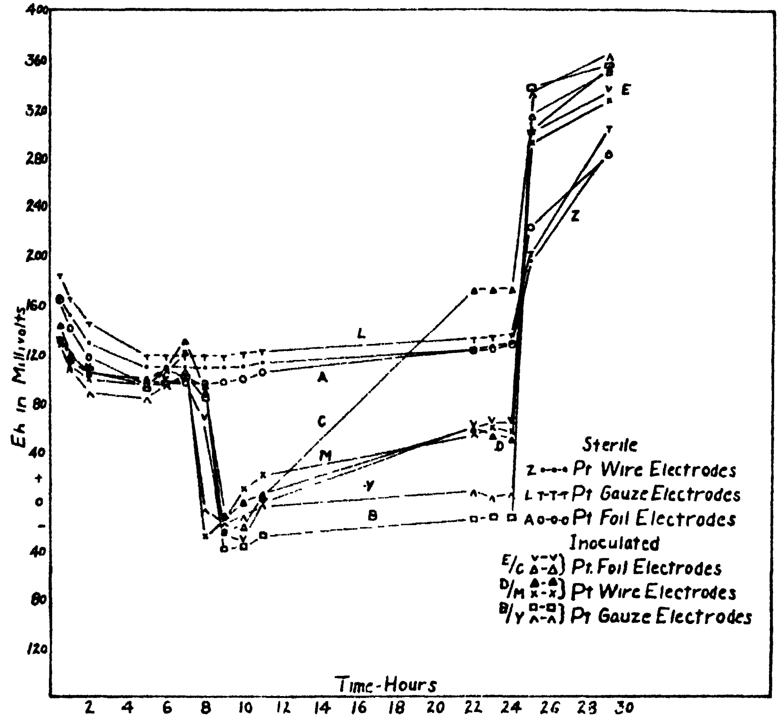


FIG. 6. TIME-POTENTIAL CURVES OBTAINED FROM THE BASIC SYNTHETIC MEDIUM UNDER ANAEROBIC CONDITIONS

Each curve represents an average of the readings obtained from duplicate electrodes in the same vessel. Pure oxygen was bubbled through the cultures beginning with the twenty-four hour period.

allowed for temperature equilibration. This was ample as judged by a control vessel. Time was counted from the end of this period.

Results obtained are plotted in figure 6 and ΔE values are given in table 1. It is to be noted, in contrast to aerobic determinations, that electrode values were regular for the first seven hours. After this time a rather sudden drop toward negative potentials occurred. This latter phenomenon was practically constant as to time and rapidity of appearance.

TABLE 1

ΔE values in millivolts for basic synthetic medium anaerobic cultures

HOURS	STERILE			INOCULATED					
	Z	A	L	M	D	C	E	Y	B
½	1	3	10	1	2	3	2	2	2
1	2	2	6	3	5	3	2	0	2
2	2	2	4	2	2	2	1	0	1
5	3	2	1	0	2	0	0	0	1
6	2	2	1	0	1	0	1	0	1
7	2	2	1	1	2	0	0	0	0
8	2	1	1	4	7	1	0	12	7
9	2	1	0	6	11	1	4	7	19
10	1	2	1	10	29	1	7	3	39
11	2	0	0	11	34	2	10	2	56
22	1	0	0	10	40	1	9	29	71
23	2	0	0	2	40	1	10	25	70
24	1	0	0	5	43	2	9	28	78
25	19	0	3	0	11	8	0	26	22
29	11	7	17	3	35	1	0	25	21

The sharp rise to positive values occuring at twenty-four hours (fig. 6) was due to pure oxygen being bubbled through the cultures at this time. This introduced irregularities, especially in the sterile medium, as is shown by table 1.

DISCUSSION

The majority of workers who have studied redox potential changes in bacterial cultures have used broth or more complex media. Using synthetic media it has been difficult to obtain such reliable data as could be obtained in a broth medium.

The use of synthetic media offers numerous possibilities for clarifying growth-potential relationships; but at the same time, many difficulties present themselves. The data show many of these irregularities and methods by which they may be circumvented.

In a non-agitated synthetic medium culture it is shown that anything resembling concurring values can only be obtained following thirty-six or more hours of incubation. After this time very little growth, in comparison to the earlier hours, occurs, so the measurements are not of what actually happened during the period of active proliferation but are of the end result of the entire process. As to whether the first portion of the time potential curve is of importance there is some disagreement. Under aerobic conditions of growth the point where the curve begins to become parallel with the abscissa axis would be dependent upon the number of organisms in the inoculum, upon the amount of poising agent present in the medium (in most cases this has been an unknown and disregarded factor), upon the oxygen tension of the medium and upon the reducing capacity of the bacterial redox systems. Any negligence in the consideration of these first three factors would make all points on the curve quantitatively erroneous. Where anaerobic conditions have been maintained, the effect of an important factor, oxygen, has been removed.

Constant mechanical agitation, under aerobic conditions of growth, is shown to be of some importance in obtaining better agreement of results. This must be the consequence of better equilibration due to the removal of poisoning material and zone effects. The observations suggested that there should be a tendency for electrodes to become sluggish due to coating with cells, etc., especially in old cultures with no agitation. It is recognized, of course, that agitation increases the oxygen tension in the medium with consequently more positive values. It is, likewise, realized that under conditions of agitation the metabolism of an organism may vary in certain respects from what would be termed normal, for it has been reported that agitation tends to decrease cell size after the early logarithmic period of growth (Mudge and Smith, 1933).

In a poorly-poised medium various types of platinum electrodes gave widely different results under identical conditions. There was no great deviation when these same types were used in a poised medium and none when tested in a simple inorganic redox system. Platinum electrodes were used almost exclusively, although a few gold ones were tried from time to time with no resulting obvious advantage. There was no apparent sluggishness of platinum types as compared with gold forms. In regard to this latter point Elema *et al.* (1934), Longsworth and MacInnes (1936) and others have reported the gold electrode to be more sensitive than platinum. This probably depends to some extent upon the type, as well as the amount, of electromotively active materials present in solution. An oxygen electrode effect would seem to account for the difference in values obtained with platinum electrode types.

It is shown that in order to obtain regular values during the growth of organisms in synthetic media under aerobic conditions, some oxidation-reduction system must be introduced which will act as an intermediate between the organisms or their products and the electrodes. This introduces a problem of some complexity for the proper substance must be chosen according to its E'_0 value and its toxicity. The amount added is also of prime importance since an excess may easily poise the potential in one region and thus give no indication as to what is actually happening as the organisms grow. Any one mediator can be good for only a limited Eh range. In regard to the latter point Kluyver and Hoogerheide (1936) have suggested the use of a mixture of oxidation-reduction dyes. There are many who would question the use of dyes and other compounds from the standpoint of toxicity but if an organism can be shown to be unaffected in growth or morphology by such substance there should be no valid objection. Undoubtedly the better values that can be obtained aerobically by the use of nutrient broth media owe their superiority to the presence of one or more unknown substances that are acting in the same position as these known redox materials.

Anaerobically, in a poorly poised medium, very good agreement was obtained during the first seven hours of growth. At the end of this period there were large numbers of organisms as judged by turbidity. There was then a sudden tendency toward negative values and irregular results were again obtained. The rapid change in potential may be the result of a rapid pH change as suggested by Gillespie and Rettger (1936) but it is hardly reasonable to believe that such a change would occur almost instantaneously. Longsworth and MacInnes (1936) have noted that shifts occurred even when the pH was kept constant, but in no case were they as rapid as those noted here. The electrode phenomena encountered at this time are reproducible with regard to time and are apparently the result of changes occurring so rapidly that the inert electrodes are unable to establish an equilibrium. The almost concordant values, regardless of electrode types, obtained in sterile control culture vessels offer direct evidence against oxygen as an interfering agent in aerobic determinations of this type.

The fact that this bacterial oxidation-reduction system is oxygen labile (and certainly many, if not all, such bacterial systems would fall into this category), introduces another very important aspect into its measurement. How can it be said that a given organism establishes a characteristic potential in any medium if those systems responsible for the potential are exposed to the air previous to, and at the time of, measurement? The value obtained may stand the test of repetition by the initial investigator but it certainly has no absolute significance since it is dependent upon a number of factors over which no control has been exerted. An anaerobic or closed system would seem to be of prime importance.

pH changes have been thought by some investigators to be the source of some irregularities. The end pH value in the broth medium was in the alkaline range of pH 7.2 and that of the synthetic medium was in the acid range of pH 4.5. This latter value was obtained in both poorly poised and semi-poised media. Hence, it is difficult to reconcile the irregularities as being due solely to pH changes during growth. There is, as yet, a lack of sufficient knowledge of the oxidation-reduction systems involved to warrant an attempt at making corrections for pH changes.

Many factors undoubtedly play important rôles in time-potential relationships of bacterial cultures. The data presented in this paper bring forth certain neglected aspects of the problems involved in the measurement of the apparent oxidation-reduction potentials established by bacteria when growing in synthetic media.

SUMMARY

In this study it is shown that a number of factors, neglected by other workers, are of importance in the measurement of apparent oxidation-reduction potentials in bacterial cultures.

The accuracy of results obtained from broth and synthetic medium cultures of *Escherichia coli* are compared. It is demonstrated that more comparable results are obtained in the broth medium. This fact is apparently due to the inherent presence of poising materials in the broth medium for when such materials are added to the synthetic medium, comparable results are likewise obtained.

The necessity for controlling the accessibility of oxygen to the culture medium is considered to be a factor influencing the magnitude of time-potential values. This is true because many if not all these redox systems are oxygen labile. Oxygen would, in such cases, affect the ratio, Ox./Red., upon which the potential determinations are dependent.

It is shown that zone effects, which are considered to result from temporary poisoning of the electrodes with bacteria or gas bubbles, account for slight variations. Agitation of the culture medium obviates these difficulties.

The type of bright platinum electrode used is only a factor for consideration when determinations in poorly poised media

are made in the presence of air. Under such conditions oxygen electrode effects are probably encountered.

The author wishes to express his gratitude to Drs. Stewart A. Koser and Dan H. Campbell for their interest and helpful criticism during the course of the work and in the preparation of the manuscript.

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SOME EFFECTS OF ASSOCIATION AND COMPETITION ON ACETOBACTER

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Received for publication February 8, 1938

I. INTRODUCTION

It is well known that one microorganism may inhibit or stimulate the growth and activity of another when the two are grown in association. Although such phenomena were early recognized, the nature of this effect is not definitely known in many instances. This competition or association may be due to the influence of the microorganisms themselves upon one another; to the production of metabolic products; to the effect of substrates or to other factors. The general aspects of association of microorganisms appear in reviews of the literature by DeBary, 1879; Ward, 1899; Lafar, 1904–07; Kruse, 1910; and more recently by Buchanan and Fulmer, 1930; Gause, 1934; Waksman, 1937; Porter and Carter, 1938 and others.

The competition between acetic-acid bacteria and yeasts in fruit juices and other fermentable solutions, which results in rapid acetification and inhibition of alcoholic fermentation, is an example of the general phenomenon. Although this competition has been widely recognized (Lafar, 1904; Kruse, 1910; Cruess, 1912; Lamb and Wilson, 1923; Buchanan and Fulmer, 1930 and others) the factors influencing rapid acetification, such as the species of yeasts and acetic-acid bacteria, the relative populations of the competing organisms, the chemical and physical conditions of the substrate are not well known. Further general observations on this type of association are presented in this paper.

II. EXPERIMENTAL

A. Source of cultures

Twenty-seven strains of *Acetobacter* isolated from commercial samples of Muscat grape musts (*Vitis vinifera* var. Muscat of Alexandria) in which the alcoholic fermentation was inhibited in its initial stages by rapid acetification were studied. These strains closely resemble *Acetobacter aceti* as defined by Visser 'T Hooft, 1925.

The ability of the cultures to attack sugars, glucosides, alcohols and the salts of organic acids was determined by inoculation into a basal medium containing 0.5 per cent tryptone (Difco), 0.2 per cent yeast-extract powder (Difco) and 0.1 per cent K_2HPO_4 in the presence of the substance to be tested. The medium was adjusted to pH 6.8–7.0 before sterilization at 15 pounds pressure for 15 minutes. Abundant growth and change in the reaction of the medium were taken as criteria of the utilization of the compounds tested. The distinguishing biochemical characters of the cultures are shown in table 1.

Glucose was the only one of the sugars or glucosides tested which was utilized. No production of calcium oxy-gluconate was noted even after 1 month. All strains produced an off-flavor known as "mousiness" when grown in sterile grape juice or grape concentrate media. The production of this characteristic "mousey" off-flavor heretofore has apparently been noted only as a characteristic of bacteria of the genus *Lactobacillus* associated with the spoiling of wines. (Müller-Thurgau and Osterwalder, 1913; Douglas and Cruess, 1936, and Arena, 1936, and others.)

The following known species of yeasts and bacteria were also used:

Saccharomyces ellipsoideus, strains No. 66, "Burgundy" and "Champagne"; *Hansenula* (*Willia*) *anomala,* American Type Culture No. 2577; *Hansenula* (*Willia*) *saturnus,* American Type Culture No. 2579; *Zygosaccharomyces priorianus* from Dr. H. H. Hall of the U.S.D.A. and *Schizosaccharomyces octosporus* from Dr. C. B. van Niel of the Hopkins Marine Station, all from the Division of Fruit Products Culture Collection.

Acetobacter aceti 4920, 4969, *A. pasteurianum* 6033, *A. xylinum* 4939, *A. suboxydans* 621, and *A. peroxydans* 838 were obtained from the American Type Culture Collection; *A. acetic* 612, *A. pasteurianum* 613, *A. xylinum* 1375, *A. acetosum* 2224, *A. suboxydans* 3734 and *A. keutzingianum* 3924 from the National Collection of Type Cultures and *A. melangenum* (Kluyver strain) from Dr. C. B. van Niel.

TABLE 1

Biochemical characters of the 27 Acetobacter cultures

CHARACTER	INCUBATION PERIOD	
	2 days	7 days
	Number of cultures giving positive reactions	
Catalase*	27	27
Growth in Hoyer's solution†	Apparent after 2 weeks incubation	
Nitrate reduction*	‡	18
Acid production from sugars:*		
Glucose	0	27
Acid production from alcohols:†		
Ethyl	27	27
Propyl	0	27
Decomposition of organic acid salts:*		
Acetic	0	23
Malonic	22	26
Malic	26	27
Citric	0	11
Tartaric	24	27
"Mousey" off-flavor in grape juice media and musts:*	‡	27 (5 to 7 days)
Brown pigment on wort agar slants*	Not observed in 1 month	

* Incubation at 37°C.

† Incubation at 30°C.

‡ Not determined.

Lactobacillus pentoaceticus, *L. mannitopeus* and *L. gayonii* obtained from the University of Wisconsin; *L. lycopersici*, *L. fructovorans* and *L. gracilis* from Dr. C. S. Pederson[1] and a species, tentatively recognized as *L. hilgardii*, from the Division of Fruit Products Collection were used.

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[1] For the most recent view concerning the classification of these species see Pederson (1938).

B. *Observations on associative growth and rapid acetification*

Grape concentrate (70°–80° Balling) was diluted with water to make a medium having a sugar content of approximately 20 per cent (20° Balling). This medium was dispensed in 75–80 cc. portions in 130 cc. bottles, which were then plugged with cotton and sterilized. It contained 0.012 gram volatile acid calculated as acetic and 0.300 gram total acid calculated as tartaric in 100 cc., determined by the methods of the Association of Official Agricultural Chemists (1930).

A 1 cc. inoculum of yeast culture 2 to 3 days old from beer-wort broth and one 2 mm. loopful of bacterial culture from liver infusion or wort-agar slants were used singly or in association as indicated in the following experiments.

a. Association of Acetobacter strains and yeasts. The associative action between the *Acetobacter* cultures and the various species of yeasts was determined by the volatile acid produced (table 2). It will be noted that marked volatile acid production occurred when the *Saccharomyces ellipsoideus* strains were grown in association with the *Acetobacter* cultures and was especially high with the "Champagne" strain. A lesser amount of volatile acid was observed with the cultures of *Zygosaccharomyces priorianus* and *Schizosaccharomyces octosporus*. With *Hansenula anomala* and *Hansenula saturnus* even less acetification occurred.

At the incubation temperature of 37°C. fermentative activity of the yeasts used in these studies is markedly below that at their optimum temperature of approximately 27°C. This factor is in part responsible for the rapid acetification noted when the various yeasts and bacteria were grown in association. The differences noted between the cultures of *Zygosaccharomyces priorianus, Schizosaccharomyces octosporus, Hansenula anomala* and *Hansenula saturnus* and those of *Saccharomyces ellipsoideus* are probably due to the lower rates at which the former ferment sugar as well as to differences in the nature of the intermediate products and the optimum temperatures for growth and activity.

b. Effect of incubation temperature on associative growth and acetification. In commercial practice rapid acetification of fermenting musts is favored by high temperatures and the cessation of yeast fermentation is generally due to the effect of temperature as well as the high acetic acid content. When naturally fermenting musts are properly cooled during the process of fermentation, the production of volatile acid is usually very slight. Therefore it was thought likely that the temperature had a marked effect on acetification in the associative action of yeasts and the *Acetobacter* cultures. To determine the effect of tem-

TABLE 2

*Volatile acid production by 27 Acetobacter strains grown in association with various yeasts**

VOLATILE ACID PRODUCED (AS ACETIC)	NUMBER OF ACETOBACTER CULTURES PRODUCING VOLATILE ACID IN PRESENCE OF:						
	S. ellipsoideus			*H. anomala*	*H. saturnus*	*Z. priorianus*	*S. octosporus*
	66	"Burgundy"	"Champagne"				
grams/100 cc.							
0-0.099	1			8	27	3	7
0.100-0.199	4	4	1	12			5
0.200-0.299	1	1	2	7		3	1
0.300-0.399	2	5				3	4
0.400-0.499	6	3				10	4
0.500-0.599	4	4				4	4
0.600-0.699	4	3				3	2
0.700-0.799	3	1	3			1	
0.800-0.899	2		1				
0.900-0.999		2	3				
1.000-1.099		1					
1.100-1.199		3	2				
1.200-1.299			1				
1.300-1.399			3				
1.400-1.499			2				
1.500-1.599			3				
1.600-1.699			1				
1.700-1.799							
1.800-1.899							
1.900-1.999			3				
2.000-2.099			1				
2.100-2.199			1				

* Incubation at 37°C. for 3 days.

perature on volatile acid production *Saccharomyces ellipsoideus* No. 66 was grown in association with each of the 27 *Acetobacter* strains at 25°, 31°, 37°, and 42°C. for a period of 3 days. Inoculations were made in quadruplicate and one of each combined culture was incubated at each temperature (table 3). Known

TABLE 3

Effect of temperature of incubation on acetification

YEAST 66 WITH ACETOBACTER NUMBER	TEMPERATURE*			
	25°C.	31°C.	37°C.	42°C.
	Volatile acid—grams/100 cc. as acetic			
68	0.360	0.465	0.741	0.239
69	0.288	0.429	0.771	0.192
73	0.279	0.252	0.571	0.114
83	0.562	0.321	0.546	0.184
84	0.233	0.498	0.551	0.110
85	0.258	0.465	0.609	0.140
86	0.145	0.270	0.402	0.158
88	0.203	0.543	1.164	0.176
90	0.204	0.498	0.648	0.098
92	0.180	0.186	0.300	0.260
98	0.189	0.477	0.903	0.286
99	0.165	0.228	0.468	0.256
100	0.621	0.588	0.780	0.238
101	0.276	0.183	0.633	0.246
102	0.147	0.213	0.537	0.180
108	0.180	0.282	0.405	0.036
109	0.102	0.306	0.477	0.268
110	0.136	0.117	0.171	0.175
111	0.099	0.303	0.591	0.162
112	0.153	0.330	0.618	0.138
113	0.153	0.149	0.606	0.535
114	0.183	0.333	0.483	0.480
115	0.150	0.172	0.378	0.220
116	0.297	0.177	0.516	0.225
117	0.165	0.306	0.510	0.326
118	0.168	0.168	0.450	0.381
119	0.105	0.180	0.324	0.305
A.T.C.C. 4920† . . .		0.180	0.068	
No. 66 alone.	0.050	0.054	0.069	0.021
Blank	0.012	0.012	0.012	0.012

* Incubation period of 3 days.

† Volatile acid not produced by other type *Acetobacter* strains.

Acetobacter species were also grown in association with yeast No. 66 at 31° and 37°C. Under the conditions of these experiments incubation at 37°C. resulted in the highest volatile acid production by the Muscat strains of *Acetobacter*. It is of interest that none of the known species of *Acetobacter* produced appreciable amounts of volatile acid when grown with the yeast at an incubation temperature of 37°C. and only one strain, *Acetobacter aceti* A.T.C.C. 4920, was capable of doing so at 31°C.

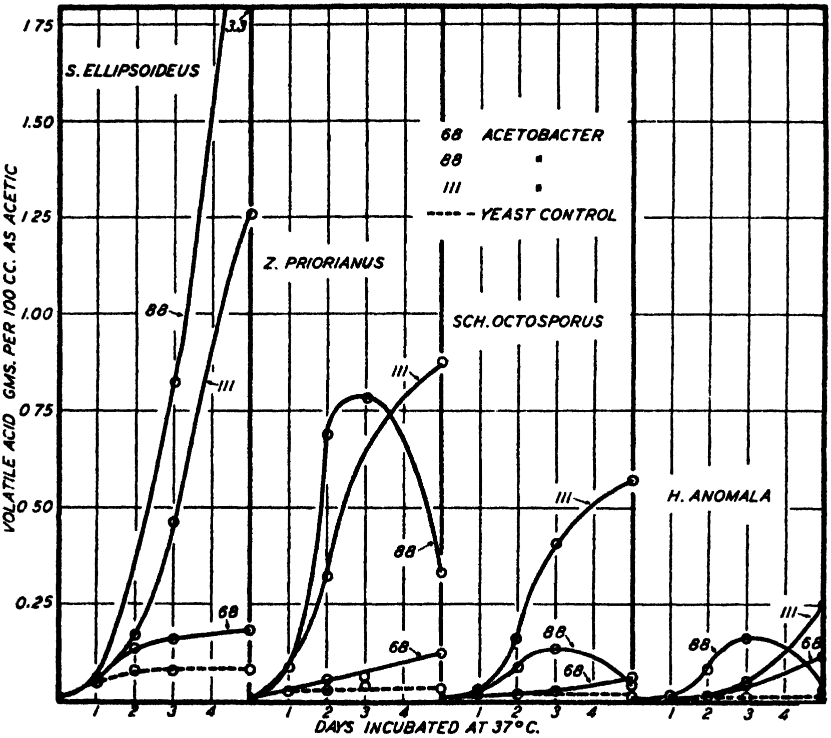

FIG. 1

c. Observations on rate of acetification. The rate of acetification caused by the 27 *Acetobacter* strains grown with various yeasts was found to depend upon the type of yeast and strain of bacterium. Furthermore a constant difference in the total volatile acid produced as a result of this association was observed when cultures were incubated for a given period of time.

The rates of acetification produced by the *Acetobacter* cultures in the presence of *Saccharomyces ellipsoideus* No. 66, *Zygosaccharomyces priorianus*, *Schizosaccharomyces octosporus* and *Hansenula anomala* were determined from the volatile acid produced after 1, 2, 3 and 5 days incubation at 37°C. Typical results are shown in figure 1.

The rates of acetification varied both with the strain of bacterium and the species of yeast. Two typical acetification rates were observed with the bacteria growing with *Saccharomyces ellipsoideus* whereas three distinct rates were noted when the bacteria were grown with the other species of yeasts.

C. Observations of association of Lactobacillus and Acetobacter

Bacteria of the genus *Lactobacillus* are frequently found growing with strains of yeast and *Acetobacter* in fermenting fruit juices undergoing rapid acetification. Under these conditions competitive growth is to be expected. Therefore the competitive effects of association on volatile acid production by heterofermentative strains of *Lactobacillus* and *Saccharomyces ellipsoideus* No. 66 were studied.

To insure rapid growth of the cultures of *Lactobacillus* when inoculated into the grape concentrate medium used in these studies, a 1 cc. portion of young liver infusion or tomato juice-tryptone broth culture was used as an inoculum when the bacteria were grown with yeast No. 66. Volatile acid determinations were made after 3 and 5 days incubation at 37°C. The results are shown in table 4.

It will be noted that all of the strains of *Lactobacillus*, with the exception of *L. fructovorans* and *L. gracilis*, produced significant quantities of volatile acid after 3 days incubation. *L. fructovorans* did not produce a large quantity of volatile acid even after incubation for 5 days, although on longer incubation the amount of volatile acid materially increased. A definite decrease in the amount of volatile acid formed was noted when the species of *Lactobacillus* were grown in association with *Saccharomyces ellipsoideus* No. 66.

Volatile acids were also produced when the *Acetobacter* strains were grown in the presence of these same lactobacilli. Although *Acetobacter* strains 68 and 111 did not significantly influence the

TABLE 4

Effect of association on volatile acid production by species of Lactobacillus

ORGANISMS	BACTERIA ALONE		BACTERIA WITH YEAST NO. 66	
	Volatile acid production (grams per 100 cc. as acetic)			
	3 days*	5 days	3 days	5 days
L. mannitopeus	0.244	0.350	0.108	0.120
L. gayonii	0.180	0.176	0.144	0.168
L. pentoaceticus	0.115	0.160	0.120	0.120
L. lycopersici	0.344	0.448	0.170	0.204
L. fructovorans	0.014	0.029	0.077	0 092
L. gracilis	0.067	0.196	0.084	0.105
L. hilgardii	0.184	0.298	0.091	0.104
S. ellipsoideus (No. 66 alone)			0.076	0.071
Uninoculated control	0.009	0.011	0.012	0.012

* Incubation at 37°C.

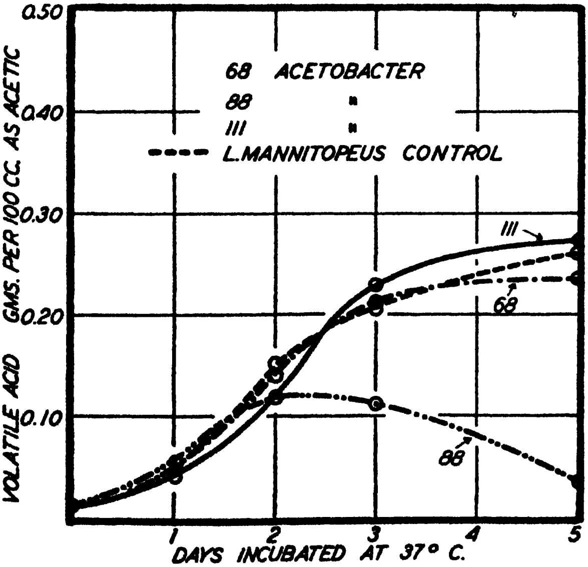

FIG. 2

amount of volatile acid produced by the species of *Lactobacillus*, *Acetobacter* 88 caused a definite decrease in volatile acid. The curves in figure 2 showing the rates of acetification with *Lactobacillus mannitopeus* grown in association with various *Acetobacter* strains are typical of the differences in acetification found for these competing bacteria. A comparison of the curves in figure 1 and figure 2 indicates a striking similarity between those observed when certain of the yeasts were grown in association with *Acetobacter* 88 and the one noted when *L. mannitopeus* was grown with the same acetic bacterium. The observations suggest that the differences in behavior of these *Acetobacter* strains is due, in part at least, to differences in their oxidative activity.

III. SUMMARY

Acetobacter cultures which seem to be similar in their characteristics from the standpoint of detailed laboratory study react differently when grown in association with other organisms. The power of rapid acetification is not possessed by all strains of *Acetobacter* when grown in association with yeasts. The bacteria isolated from "stuck" wines all possessed this characteristic whereas well-known strains obtained from various collections were unable to bring about rapid acetification when grown with yeast at 37°C. One known strain, *Acetobacter aceti* A.T.C.C. 4920 caused a small amount of acetification when grown in association with yeast at 30°C.

The rate of acetification was influenced by the types of yeasts grown in association with the bacteria and by the strains of bacteria. Several characteristic trends in volatile acid production were observed. The rate of acetification was also influenced by the temperature of incubation.

Competition between strains of *Lactobacillus* and *Saccharomyces ellipsoideus* resulted in a decrease in the amount of volatile acid formed in 5 days at 37°C. The rates of acetification by *Lactobacillus mannitopeus* grown in association with the *Acetobacter* strains paralleled those obtained with the *Acetobacter* cultures grown in association with different yeasts.

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HEAT STERILISED REDUCING SUGARS AND THEIR EFFECTS ON THE THERMAL RESISTANCE OF BACTERIA

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Received for publication March 19, 1938

That the thermal resistance of at least some types of microorganisms is increased when heated in hypertonic solutions of various carbohydrates is now well established. A review of the literature concerning this subject has been presented by Fay (1934). This author found that sucrose and glucose in concentrations above 0.75 M were protective for *Escherichia coli.* No protection was obtained with saturated lactose solution and 50 per cent maltose solution gave only slight protection. In these experiments the bacteria were heated in nutrient broth solutions of the sugars. It was not stated by what means the solutions were sterilised.

In a later paper Baumgartner and Wallace (1934) published results which were not in agreement with Fay's findings in respect to lactose. They found that lactose in 17 per cent solution and sucrose, glucose and invert sugar in 20 per cent solutions were each protective for *Escherichia coli.* In this investigation the sugars were sterilised by autoclaving in a dry state and then dissolved in sterile buffer solution.

The effects of heat sterilisation on media containing sugars have been investigated by several workers. Mudge (1917) reported that hydrolysis of lactose and maltose was greater when sterilised in streaming steam for three successive days than when once autoclaved at 15 pounds pressure for 15 minutes. Fulmer, Williams and Werkman (1931) noted growth stimulation of yeast and certain bacterial species including *Escherichia coli* in a medium (containing 0.6 gram NH_4Cl, 0.2 gram K_2HPO_4 and 5.0 grams glucose per 100 cc.) which had been sterilised at 15 pounds pressure for 15 minutes. The stimulant was not caramel but a substance produced parallel with caramel. Previously, Lewis (1930) reported that growth of some types of bacteria was completely inhibited in media containing either glucose, maltose, lactose, galactose or levulose which together with phosphate and a source of nitrogen had been sterilised at 122°C. for 15 minutes. The reaction depended on the concentration of sugar, phosphate and nitrogen compound present and it was concluded that the inhibition was due to the conversion of the nitrogen compound into a form not suitable for assimilation by certain bacterial species.

In the course of work in this laboratory on the quantitative effects of sugars on the thermal resistance of *Escherichia coli* it was found that the method of sterilising *reducing* sugars greatly influenced the results obtained. The experiments indicated that for sugars of this type it is necessary to adopt the filtration method of sterilisation, since their heat treatment in buffer solution or nutrient broth results in the formation of material which reduces bacterial heat resistance. The toxic material was formed during heat sterilisation of the sugars in solutions with or without nitrogenous compounds. The material acts as a direct toxic agent and is not connected with alteration of nutrient materials.

METHODS

The sugars used in this work were supplied by British Drug Houses Ltd., and were the purest available. They were dissolved in McIlvaine's disodium phosphate-citric acid buffer solution or nutrient broth (both at pH 7.0). The basic thermal resistance of the test organism was estimated in plain buffer solution or nutrient broth. A suspension of a filtered, 24 hour agar growth of *Escherichia coli*[1] was suitably diluted and from a pipette graduated in 0.01 cc., exactly 0.25 cc. was mixed with 20 cc. amounts of the various solutions. These were subjected to a 1-minute uniform shaking. The control tube (without sugar) was diluted 1:1000 with sterile distilled water and then plated

[1] National Collection of Type Cultures, strain No. 86.

in duplicate. Counts were not made of the sugar solutions, equal inoculation being assumed in each instance. Three cubic centimeters amounts of each inoculated solution were then placed into sterile tubes (5 x ½-inch) and these were heated in a water bath at 54°C. for eight minutes. No differences in heat penetration into the various solutions were noted. After rapid cooling the solutions were diluted 1:100 and 1 cc. amounts of this and also the undiluted solution were plated in duplicate into 20 cc. of plain nutrient agar (pH 7.0). The amount of sugar added to the test plates via the inoculum was, in the case of the 1:100 dilution of glucose, only 0.0045 per cent, an amount which was considered unlikely to influence the results appreciably. In the plates receiving undiluted solution the concentration of glucose reached 0.45 per cent, but where such plates were used in obtaining counts the number of survivors was so low that any stimulative influence of the sugar was disregarded. The plates were incubated for 3 days at 37°C., counted and held for a further period of 4–5 days at room temperature. Counts exceeding 10,000 per cc. are given to the nearest 500.

EXPERIMENTAL

1. *The effects of heat-sterilised and filter-sterilised sugar solutions on the thermal resistance of E. coli*

The sugars were used in 0.5 M concentration. Two series of sugar solutions were tested in which the solvents were buffer solution and ordinary nutrient broth (pH 7.0). The solutions were sterilised at 112°C. for 15 minutes. Due to this treatment caramelisation and acid formation occurred to a varying degree in each of the reducing sugar solutions. After sterilising, the pH values of the solutions were adjusted to 7.0 with sterile N/10 NaOH solution.

Table 1 gives results with buffered solutions and broth solutions of the sugars. These form two separate experiments but are included in one table for convenience of presentation.

The results showed that in each instance, autoclaved solutions of reducing sugars gave an increased lethal effect. This was also apparent when fractionally sterilised sugar solutions were used (table 2). In this case, sugar solutions in buffer were sterilised in streaming steam for 30 minutes on 3 successive days. Changes in pH which occurred in the reducing sugar solutions were adjusted after sterilising.

These effects, in which heat sterilised reducing sugar solutions increased the thermal destruction of *E. coli*, did not occur if the solutions were sterilised by filtration. With this method of sterilisation, all the sugars tested increased the number of cells surviving heat treatment (see table 3). In this experiment a Seitz Filter fitted with Seitz E. K. filter pads was used. Heat treatment of the solutions was completely avoided.

TABLE 1

Survival of E. coli exposed for 8 minutes at 54°C. in autoclaved sugar solutions

HEATING MEDIUM	NUMBER OF VIABLE CELLS PER CC.	
	Before heating	After heating
Plain buffer (control)	232,000	3,200
0.5 M Glucose in buffer	232,000	184
0.5 M Galactose in buffer	232,000	104
0.5 M Lactose in buffer	232,000	210
0.5 M Maltose in buffer	232,000	0
0.5 M Sucrose in buffer	232,000	21,500
0.5 M Mannitol in buffer	232,000	10,500
0.5 M Glycerol in buffer	232,000	7,500
Plain broth (control)	224,000	16,000
0.5 M Glucose in broth	224,000	6,000
0.5 M Galactose in broth	224,000	4,200
0.5 M Lactose in broth	224,000	3,500
0.5 M Maltose in broth	224,000	7,000
0.5 M Sucrose in broth	224,000	33,500
0.5 M Mannitol in broth	224,000	24,000
0.5 M Glycerol in broth	224,000	21,000

Lewis (1930) observed that solutions of glucose in distilled water autoclaved at 122°C. did not cause growth inhibition when incorporated in a nutrient medium.

We have noted that for the production of toxic material from solutions of glucose in distilled water, it was necessary to heat the solution until caramelisation occurred. This requires treatment

TABLE 2

Survival of E. coli exposed for 8 minutes at 54°C. in fractionally-sterilized sugar solutions

HEATING MEDIUM	NUMBER OF VIABLE CELLS PER CC.	
	Before heating	After heating
Plain buffer (control)	420,000	8,500
0.5 M Glucose in buffer	420,000	2,100
0.5 M Galactose in buffer	420,000	120
0.5 M Lactose in buffer	420,000	30
0.5 M Maltose in buffer	420,000	256
0.5 M Sucrose in buffer	420,000	25,000
0.5 M Mannitol in buffer	420,000	21,000
0.5 M Glycerol in buffer	420,000	18,500

TABLE 3

Survival of E. coli exposed for 8 minutes at 54°C. in filter-sterilized sugar solutions

HEATING MEDIUM	NUMBER OF VIABLE CELLS PER CC.	
	Before heating	After heating
Plain buffer (control)	272,000	5,100
0.5 M Glucose in buffer	272,000	14,500
0.5 M Galactose in buffer	272,000	11,500
0.5 M Lactose in buffer	272,000	49,000
0.5 M Maltose in buffer	272,000	13,500
0.5 M Sucrose in buffer	272,000	32,500
0.5 M Mannitol in buffer	272,000	15,000
0.5 M Glycerol in buffer	272,000	10,000
Plain broth (control)	280,000	11,000
0.5 M Glucose in broth	280,000	22,000
0.5 M Galactose in broth	280,000	22,500
0.5 M Lactose in broth	280,000	33,000
0.5 M Maltose in broth	280,000	20,500
0.5 M Sucrose in broth	280,000	28,000
0.5 M Mannitol in broth	280,000	25,000
0.5 M Glycerol in broth	280,000	18,500

far in excess of that necessary when buffer or broth is used. Heat treatment of the same order as applied to buffered or broth solutions does not produce caramelisation in distilled water solutions of glucose and solutions so treated are not toxic for *E. coli* at

54°C. On the other hand, if distilled water solutions of glucose are heated until caramel is formed, they increase the rate of thermal destruction of the organism (table 4). The solutions of glucose in distilled water were heated in large quartz tubes. In the case of the solution autoclaved for 15 minutes at 112°C., no change in colour occurred but the solution heated for 3 hours at 130°C. became straw coloured and acid was formed. The solutions were evaporated *in vacuo* (210 mm. Hg) until the sugar remained as a very thick syrup (about 80 per cent glucose). The sugar tended to char if evaporated beyond this point. The syrup was then redissolved in buffer solution to give 0.5 M concentration of glucose. The solutions were finally sterilised by filtration. The distillates obtained from the evaporation proc-

TABLE 4

Survival of E. coli exposed for 8 minutes at 54°C. in distilled water-treated glucose solutions

HEATING MEDIUM	TREATMENT	NUMBER OF VIABLE CELLS PER CC.	
		Before heating	After heating
1. Plain buffer (control)		254,000	4,500
2. 0.5 M Glucose in buffer	Sterilised at 112°C. for 15 minutes	254,000	704
3. 0.5 M Glucose in buffer	Filter-sterilised	254,000	12,500
4. 0.5 M Glucose in buffer	Heated in distilled water at 112°C. for 15 minutes; evaporated and redissolved in buffer. Solution then filter-sterilised	254,000	9,800
5. 0.5 M Glucose in buffer	Heated in distilled water at 130°C. for 3 hours; evaporated and redissolved in buffer. Solution then filter-sterilised	254,000	1,100
6. Plain buffer	Prepared with distillate from evaporation process of distilled water solution of dextrose in 4	254,000	5,800
7. Plain buffer	Prepared with distillate from evaporation process of distilled water solution of dextrose in 5	254,000	4,000

esses were used for preparing plain buffer solutions and were included in the tests with other controls.

Heat sterilisation of glucose without production of toxic material is possible if the sugar is autoclaved in a dry state and subsequently dissolved in cold sterile buffer solution. For example, in plain buffer solution, with an initial concentration of 210,000 viable cells per cubic centimeter, 4,500 cells per cubic centimeter survived heating at 54°C. for 8 minutes. In 0.5 M glucose (sugar sterilised dry for 15 minutes at 112°C. and then dissolved in cold sterile buffer) the survivors numbered 10,500 per cubic centimeter.

TABLE 5

Survival of E. coli exposed for 8 minutes at 54°C. in autoclaved dextrose solutions (2.5 M and 0.5 M concentration)

SOLUTION	TREATMENT	NUMBER OF VIABLE CELLS PER CC.	
		Before heating	After heating
1. Plain buffer	Autoclaved	560,000	6,500
2. 2.5 M Glucose in buffer	Autoclaved	560,000	350
3. 2.5 M Glucose in buffer	Filter-sterilised	560,000	120,000
4. 0.5 M Glucose in buffer	Autoclaved	560,000	720
5. 0.5 M Glucose in buffer	Filter-sterilised	560,000	26,500

Experiment did not support the hypothesis that, with more concentrated sugar solutions, the effect of toxic substances resulting from heat sterilisation would be masked by the protective effect of the increased amount of sugar. The solutions used were 2.5 M and 0.5 M glucose in buffer (pH 7.0). These were autoclaved at 112°C. for 15 minutes. It was noted that the 2.5 M solution was caramelised to a greater extent than the 0.5 M solution by this treatment, the former was deep straw, whilst the latter was pale straw in colour. Acid formed during sterilisation was afterwards neutralised with sterile NaOH solution. Controls consisted of duplicate samples, filter sterilised, of the sugar solutions and plain buffer solution.

2. The production of caramel and toxic material

Results of the experiments with distilled water solutions of glucose suggested that caramelisation is a necessary complement to the production of toxic material. If this is so, then increased caramelisation of a solution would be expected to lead to increased toxicity.

To test this theory the following experiment was made. Twenty cubic centimeter amounts of 0.5 M glucose in buffer solution (pH 7.0) were autoclaved in large test tubes, the treatment varying with each tube. A further 20 cc. of the same solution were filter-sterilised, as were 20 cc. of plain buffer solution. In the heated solutions caramelisation increased according to the severity of the heat treatment. Acid production occurred and adjustments to pH 7.0 were made with sterile NaOH solution. Results are shown in table 6.

The data given in table 6 indicate that the toxic material arising from the heat treatment of dextrose solution is produced parallel with caramel or that the caramel is in itself toxic.

It was found that the toxicity of a caramelised, buffered glucose solution was only slightly reduced by treatment with a purified adsorbing charcoal (table 7). In this experiment, the glucose solution (0.5 M in buffer) was autoclaved for 20 minutes at 120°C. This treatment caused the solution to become dark brown in colour and acid was formed. After treatment with charcoal for 60 minutes at 37°C. the solution was passed through a Seitz filter. The solution was then only faintly coloured, practically all the caramel having been removed. After filter-sterilising the solution was neutralised with sterile NaOH solution.

The data given in table 7 indicate that almost complete removal of caramel from a heated glucose solution has only a very small effect on the toxic strength of the solution. It would thus appear that caramel is not the toxic agent. The slight decrease in toxicity which occurred upon removal of caramel was possibly due to adsorption of a small amount of the toxic material simultaneously with caramel.

TABLE 6

Survival of E. coli exposed for 8 minutes at 54°C. in dextrose solutions caramelised to a varying degree

SOLUTION	TREATMENT	COLOUR (INDICATING EXTENT OF CARAMELISATION)	NUMBER OF VIABLE CELLS PER CC.	
			Before heating	After heating
1. Plain buffer	Filter-sterilised		360,000	4,500
2. 0.5 M Glucose in buffer	Filter-sterilised		360,000	24,000
3. 0.5 M Glucose in buffer	1 minute at 112°C.	No change (as in 1 and 2)	360,000	7,500
4. 0.5 M Glucose in buffer	15 minutes at 112°C.	Pale straw	360,000	1,400
5. 0.5 M Glucose in buffer	60 minutes at 112°C.	Deep straw	360,000	0
6. 0.5 M Glucose in buffer	60 minutes at 120°C.	Dark red-brown	360,000	0

TABLE 7

Survival of E. coli exposed for 8 minutes at 54°C. in heated dextrose solutions before and after adsorption with charcoal

SOLUTION	TREATMENT	NUMBER OF VIABLE CELLS PER CC.	
		Before heating	After heating
1. Plain buffer (control)	Filter-sterilised	420,000	3,800
2. 0.5 M Glucose in buffer.	Autoclaved 20 minutes at 120°C.	420,000	0
3. 0.5 M Glucose in buffer.	Autoclaved for 20 minutes 120°C.; treated with charcoal then filter-sterilised	420,000	120
4. 0.5 M Glucose in buffer	Not heated, treated with charcoal, then filter-sterilised	420,000	23,000

In connection with the adsorption of caramel, it may be stated that colorimetric tests failed to reveal any marked removal of caramel from a heated glucose solution after treatment with massive doses of washed cells of *E. coli.*

3. *Effects of heat-sterilised glucose broth on the growth of E. coli*

In addition to the toxicity of heat-sterilised glucose nutrient broth demonstrated in the thermal resistance tests (table 1), it has been found that such a medium is also capable of retarding the growth of *E. coli* (table 8). Lewis (1930) found that the production of inhibitory media was affected by the phosphate content of the medium, at least 0.2 per cent K_2HPO_4 being required for inhibition to occur. He also found that a glucose medium which was inhibitory for some species did not inhibit the growth of *E. coli*. It would seem, however, that Lewis's experiments were concerned with complete inhibition rather than the slight effects considered in the present report.

TABLE 8

The growth of E. coli in heat-sterilised and filter-sterilised glucose-nutrient-broth

MEDIUM	TREATMENT	TIME FOR GROWTH TO BECOME MACROSCOPICALLY VISIBLE	GROWTH AFTER 24 HOURS	GROWTH AFTER 7 DAYS
		hours		
1. 0.5 M Glucose broth	Autoclaved	5½	+	+++
2. 0.5 M Glucose broth	Filter-sterilised	4	++	++++
3. 0.5 M Glucose broth, 0.5 per cent K_2HPO_4	Autoclaved	6½	∓	+++
4. 0.5 M Glucose broth, 0.5 per cent K_2HPO_4	Filter-sterilised	4	++	++++

++++ = maximum growth.

In the heated glucose nutrient-broth which we have noted to be toxic at 54°C. and also slightly inhibitory to the growth of *E. coli* at 37°C., the only phosphate present was that occurring normally in the medium, about 0.06 per cent calculated as K_2HPO_4. Experiments were made in which the phosphate content of the medium was increased by addition of K_2HPO_4. The heated media were sterilised at 112°C. for 15 minutes. Changes in pH occurred during sterilisation, and the media showed signs of caramelisation; that containing added phosphate being more affected than that without added phosphate. After sterilising, the pH of the heated media was adjusted to the original value of 7.0 with sterile NaOH solution. The control media (pH 7.0) were sterilised by filtration. To 20 cc. of each of the media prepared, was added one drop of a filtered distilled water suspension of *E. coli* and the tubes incubated at 37°C. Before inoculation all tubes were brought to a temperature of 37°C. by immersion in a water-bath for 20 minutes.

It will be seen that the growth of *E. coli* is delayed in heat-sterilised glucose broth. Increasing the phosphate content of the medium appears to increase the delay. The delay persists throughout the growth of the culture and in view of the increased thermal destruction in similar media (table 1) it is reasonable to suppose that it is due to direct toxic action on the bacteria of a substance produced during sterilisation of the medium. Topley and Wilson (1929), discussing the growth of bacteria in unsuitable media state: "In a medium that is unfavourable—for example one with too high a hydrogen ion concentration—the lag phase is often greatly prolonged; several hours may elapse before the organism begins to grow, but once it has started, it may grow as well as in a medium of optimum reaction. This statement is only true within certain limits; if the medium is very acid, then the growth may not only be slow to begin, but may remain poor in its later stages. A similar observation has been made by Moore (1915) relative to the growth of pneumococci in a medium containing a high dilution of disinfectant."

4. Preliminary chemical investigation of heat-sterilised glucose solutions[2]

A preliminary chemical investigation into the nature of the toxic material has been made. A solution of 0.5 M glucose in buffer solution was autoclaved for 15 minutes at 112°C. which treatment caused caramelisation, rendering the solution straw coloured. This solution was steam distilled from acid solution, but the distillate was negative to tests for acid and aldehyde; the distillate did not decolorize bromine water and no colouration was obtained on adding $FeCl_3$.

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[2] I am indebted to Dr. H. K. Dean of this laboratory for carrying out this portion of the work.

With a similar solution of sugar heavily caramelised, by autoclaving for 3-hours at 120°C., steam distillation from acid solution resulted in an acid distillate in which aldehyde was detected by means of Schiff's reagent. The distillate reduced $HgCl_2$ and decolorized bromine water. Tests for oxalic acid in the original sugar solution and for formic acid in the distillate were negative. On treatment of the distillate with $FeCl_3$ a purplish colouration was obtained which was not phenolic in origin and may have been due to the presence of a keto-enol substance. Pyruvic acid was not present.

A solution of glucose in distilled water, slightly caramelised by heating at 130°C. for 3-hours, gave no precipitate on addition of lead acetate solution.

DISCUSSION

The object of this investigation was to ascertain the effects on the thermal resistance of *E. coli* of buffered and nutrient broth solutions of reducing sugars sterilised by various methods. The data collected indicate that the filtration method of sterilisation should be adopted for such work. Using this method, all the sugars tested, including lactose and maltose, increased the number of cells surviving heat treatment. It thus seems that conflicting reports in the literature concerning the effects of certain sugars on bacterial heat resistance may be due to the use of different methods of sterilising the sugar solutions.

Search of the literature has so far failed to reveal any conclusive account of work which would throw light on the nature of the toxic material formed by the thermal treatment of reducing sugars, in solution (excluding distilled water). The so-called caramel is known to be a highly complex mixture containing colloidal constituents (Clayton, 1932) but caramel as such has been excluded by the necessary tests. It would appear, therefore, that along with caramelisation, some other chemical compound is produced, which in the pure state must be highly toxic to bacteria.

Lewis (1930) discussed the literature concerning the transformations which occur in sugars under the influence of heat and various compounds such as phosphates and caustic alkalies, and concluded that the material causing growth inhibition in his experiments was an aldehyde: This aldehyde combined with the nitrogen source in the medium and rendered it non-assimilable. Ambler and Byall (1935) have reported extensive data on the influence of non-sugars on the caramelisation and inversion of sucrose, drawing attention to the deep-seated and complex isomerisations and degradations involved. Unfortunately, their results cannot be applied to the investigations just described.

Further work is obviously desirable in an attempt to isolate and identify the toxic agent. This should be attempted by working with greater bulk and investigating the fractions obtained by adsorption technique. Removal of colloidal complexes should be first effected, e.g., with alumina cream. If the toxic agent has an affinity for protein it may be expected to reveal this by surface film studies in a Langmuir-Adam trough. It is also necessary to ascertain whether the toxic effect is general for a wide range of bacteria.

SUMMARY

Sterilisation of reducing sugar solutions by autoclaving at 112°C. for 15 minutes or steaming for 30 minutes on three successive days results in the formation of material which is directly toxic for *Escherichia coli* at 54°C. and is capable of delaying the growth of this organism at 37°C.

The material is produced simultaneously with caramel in reducing sugar solutions in which the solvent is McIlvaine's citric acid-phosphate buffer or nutrient broth; for its production in distilled water solution, drastic heat treatment, sufficient to cause caramelisation is required. The toxic material does not occur when buffered or broth solutions of reducing sugars are sterilised by filtration.

Filter-sterilised glucose, galactose, lactose, maltose, sucrose, mannitol, and glycerol in 0.5 M concentration in buffer or broth were all found to protect *Escherichia coli* against thermal destruction.

A preliminary chemical investigation failed to reveal the nature of the toxic material.

I wish to thank Dr. William Clayton for his helpful criticism and advice.

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A MICROORGANISM DECOMPOSING GROUP-SPECIFIC A SUBSTANCES

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Received for publication March 29, 1938

The use of bacteria to effect the degradation of serologically active carbohydrates was initiated by Avery and Dubos (1930; see also Dubos and Avery 1931) in studies on the capsular carbohydrate of Pneumococcus Type III; and other microorganisms capable of splitting polysaccharides of bacterial origin have since been found. The present report concerns the isolation and cultural characters of an apparently hitherto undescribed bacterium, having specialized food requirements and not growing on most common culture media, which is able to decompose actively the group-specific substance found in the saliva of human beings of blood group A and in various group A preparations obtained from animal materials, namely horse saliva (Landsteiner 1936) commercial pepsin and gastric mucin (Landsteiner and Chase 1936). From chemical investigations it has become probable that the substances underlying the serological differentiation of human blood groups are of polysaccharide nature (for literature, see Landsteiner 1936, Landsteiner and Chase 1936). Apart from adding to the relatively small number of organisms known to attack "specific" polysaccharides—a property not as common as might have been supposed—the description of a new species with unusual characters may be of interest.

Of bacteria which decompose serologically active complex polysaccharides, mention may be made of the following: the *Myxococcus* of Morgan and Thaysen which acts upon several specific polysaccharides of bacterial origin (Morgan and Thaysen 1933; Meyer and Morgan 1935) and also "group specific" A substances (Landsteiner and Chase 1935a); strains of bacteria, isolated by Sickles and Shaw, which can degrade different pneumococcal carbohydrates (Sickles and Shaw 1934, 1935) and of which one, *Saccharobacterium ovale*, acts on group A substances as well (Landsteiner and Chase 1935b); and *Clostridium welchii* found by Schiff (1935) to attack group A substances. With regard to the decomposition of group A substances by other microorganisms, it may be mentioned that we found these preparations to be unaffected when incubated with a number of samples of decomposing vegetable matter; they were not altered by many common bacterial species, as Schiff (1935) has reported also, or by a chitinovorous bacterium[1] and several cellulose-decomposing organisms. In two cases, some degree of destruction of the A substance was detected after inoculation with rotting vegetables. Rather active decomposition was noted with a sample of aged, dry leaf mold and from this there was isolated the bacterium to be described.

Leaf mold was introduced into a medium comprising the A substance from commercial pepsin dissolved in the mineral base of Dubos and Avery (1931) (1 gram ammonium sulfate and 2 grams dipotassium phosphate per liter of water); after some days the culture medium had lost its specific property of inhibiting the hemolytic action (for sheep cells, in the presence of complement) of rabbit antisera to human group A erythrocytes. In isolating the organism, serial passages in 3 cc. portions of medium containing 0.02 per cent of the A preparation were employed in the beginning, but the persistence of extraneous bacteria in much greater numbers than the active organism necessitated plating on a medium containing A substance, mineral base and 1 per cent agar, the fishing of small areas of confluent growth (rather than single colonies) into the fluid A medium, and continued plating of such mixtures as were found after incubation to effect destruction of the specific substrate. After a few repetitions of this procedure, colonial fishings were practicable, and yielded a pure culture of the organism. As a precaution, eight consecutive platings from single colonies were made.

[1] A culture belonging to group I, supplied by the kindness of Dr. Anne G. Benton (1935).

For maintenance and study of the strain, two types of culture media were used. The first was a mineral medium containing 0.1 per cent of preparations rich in A substance, made from commercial pepsin and gastric mucin[2] (hereafter designated as "P" or "M" respectively), or slants of 0.5–1.0 per cent A substance and 1 per cent agar in mineral medium, employed usually at pH 7.0 to 7.2 (the optimum range is pH 6.0–6.5) because of moderate acid production during the growth of the bacteria.[3] After a preliminary study of the organism a second type of medium, termed "SP", was developed, based upon a utilizable carbohydrate and proteose peptone (Difco), which supports growth, probably chiefly because it has a relatively high content of group-specific A substance. The medium consists of 1 per cent Difco proteose peptone, 1 per cent sucrose, 0.1 per cent $(NH_4)_2SO_4$, 0.2 per cent K_2HPO_4, with 1 per cent agar as desired, in tap water, adjusted preferably to pH 6.5: the sucrose was added as a sterile solution to the autoclaved base. It should be mentioned that after cultivation for over a year on this "SP" medium the organism showed some decided alteration in cultural characters, as contrasted with a line maintained on medium "M".

When first isolated in pure culture, the bacterium was transplanted to a variety of common substrates, as well as synthetic media containing ammonium lactate or asparagin and tryptophane. There was no evidence of growth except in the cases of rabbit-blood beef-infusion Witte peptone agar, and litmus milk. On the former, a few small colonies slowly developed; with continued cultivation, the organism could be adapted to a scanty, unsatisfactory growth on beef infusion Witte peptone agar, again probably because of A substance in the peptone.

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[2] The latter material, as used in the media, was prepared from crude gastric mucin (Wilson and Co., No. 1701-W) by heating a slightly acidified 2 per cent aqueous solution at 100°C., discarding material sedimenting upon centrifugation, and selecting the alcohol-insoluble fraction precipitating between $\frac{3}{4}$ and 2 volumes of alcohol, substances insoluble at the lower alcohol concentration first being removed by Berkefeld filtration; the material was reprecipitated once by addition of 2 volumes of alcohol to an aqueous solution.

[3] Whenever growth was evident in fluid media "M" or "P" (within 20 to 48 hours), degradation of the A substance could be confirmed serologically.

PURE CULTURE STUDY

General

For the determination of cultural characters, the organism was examined, when appropriate, on both "SP" and "M" media. For other substrates, 1 per cent proteose peptone in mineral medium was employed as a basal fluid, and growths on proteose peptone were used as seeding cultures. For nitrate reduction, 0.1 per cent potassium nitrate was added to this solution, while indol production by the organism was examined on the proteose peptone solution alone and with added "tryptone" or with 0.03 per cent tryptophane. Cellulose digestion was tested on fluid and solid media containing amorphous cellulose, prepared according to the method of McBeth (1916), in the peptone base or in medium "M". In making the biochemical tests, procedures recommended in the Manual of Methods for Pure Culture Study of Bacteria were followed.

Morphology

The best preparations were made from growths on fluid medium "M" at pH 5.5 to 6.5, incubated at temperatures between 25° and 32°C. for one to four days; the cells were then fairly uniform and stained clearly, the organism being a coccoid rod, occurring singly and in pairs, the somewhat oval forms measuring about 0.3–0.6μ by 0.5–0.7μ. On agar medium "SP" at pH 6.5, the individuals vary in size to a greater degree than noted above, averaging about 0.5 by 0.6μ, but large cells 0.9 by 1.2μ are also seen. On fluid medium "SP" at pH 6.5, the cell is not sharply outlined, and there is some variability in the intensity of the stain. Organisms grown in fluid media at reactions more alkaline than pH 6.5 stain increasingly poorly the higher the pH value, and rapid and complete autolysis occurs in but weakly alkaline fluids, e.g. upon treatment with dilute ammonium hydroxide.

Carbohydrate utilization

The organism did not grow on solutions of the test carbohydrates in the absence of proteose peptone. In media at pH 7.0 having 1 per cent carbohydrate and 1 per cent proteose peptone in the mineral base, a greater final amount of growth than in the peptone control occurred in the presence of glucose, lactose, sucrose, galactose, mannose, xylose, maltose, inulin and dextrin, whereas mannitol, salicin and glycerol appeared not to be utilized.[4] The rate of development of the organism was markedly retarded over that in the peptone control by the presence of mannose, xylose, or salicin. There were only inconsiderable increases in acidity, about 0.2 pH unit, with the most vigorous growths; further studies with slants (1 per cent agar) made from sugar-peptone solutions and indicators revealed the development of acidity during growth on lactose (pH 6.6) and glucose (pH 6.8), but no or only insignificant changes in reaction with sucrose, galactose and maltose. No gas could be detected in Smith tubes (or in agar stabs) during active utilization of added carbohydrates; the growth was however limited to the surface of the open arm.

CULTURAL CHARACTERS

Coccoid rods, 0.3 to 0.6 by 0.4 to 0.9 microns, occurring singly and in pairs. Non-motile. Gram-negative. No spores demonstrated.

Gelatin "M" stab: no liquefaction.

Agar colonies: On beef-extract agar, no growth. On 0.75 per cent "M", 1 per cent agar: after two days at 32°, visible colonies 0.75 mm. in diameter, increasing in size to 5–7 mm. in 12 days. Circular, smooth, slightly raised, with an opaque raised dot set in a central depression. The internal structure appears coarsely granular. By transmitted light, bluish with thin light margins, by reflected light faintly yellow.[5] The consistency is butyrous to viscid. On this medium, only, daughter colonies often arise near the periphery of the parent colony after the fifth day. On "SP" 1 per cent agar: as above, except that the colonies are opaque and somewhat smaller. On blood agar (usual beef infusion peptone base): after 17 days at 32°, 0.75 mm. diameter, circular, raised, glistening, rather white.

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[4] The utilization of the carbohydrates was confirmed by means of the Hanes' modification (1929) of the Hagedorn Jensen method for the microestimation of reducing sugars; media containing sucrose, inulin, dextrin, salicin, or lactose were first subjected to acid hydrolysis. The author wishes to thank Mr. Robert A. Harte for these determinations.

[5] When the agar concentration is higher, the colonies are smaller and quite transparent at first, but a decided brownish yellow color eventually develops.

Subsurface agar colonies: On 0.75 per cent "M", 1 per cent agar: distinctly brownish, often branched or lanceolate, opaque.

Agar stroke: On 1 per cent "M", 1 per cent agar: after 5 days at 32°–35°, moderate growth, glistening, contoured, flat, with margins rugose; translucent, slightly irridescent along margins; secondary colonial growths commonly observed along line of original streak; some irregular, cleared areas on old slant growths.

Infusion broth: no growth.

"M" or "SP" fluid media: growth at surface, settling to give an even turbidity.

Litmus milk: becomes slimy, reaction unchanged.

Lead acetate: slight browning?

No growth on potato.

Indol not produced.

Nitrates reduced.

No gas or pronounced acid production in carbohydrate media, but glucose, galactose, mannose, lactose, sucrose, maltose, xylose, inulin and dextrin are utilized. Very slight acid reaction detected with lactose and glucose.

Starch not attacked.

No characteristic odor.

Obligate aerobe; in agar shake cultures, growth does not occur more than 2 mm. below the surface.

Optimum temperature: about 32°; no growth at 40°C.

Thermal death, 2 day growth in "M" or "SP" fluids, after neutralization: 10 minutes at 51°C. not at 48°C.

Reaction of medium: pH limits of growth from 5.5 to 7.7, optimal range 6.0 to 6.5.

Cellulose agar not attacked.

Habitat: isolated from leaf mold.

CLASSIFICATION

The systematic position of a Gram-negative, non-motile, obligately aerobic, coccoid bacterium with the cultural peculiarities, as regards common sources of energy, of using certain peptones only, of growing either not at all or sparsely on common media, and—in the presence of peptone—of utilizing a number of sugars without frank production of acid or gas is difficult of decision.

A kinship with the two organisms in the genus *Saccharobacterium* erected by Sickles and Shaw (1934) in the family Mycobacteriaceae seems excluded on morphological grounds, also because of the failure of certain substances to inhibit growth when added to a favorable medium, namely the presence of 0.7 per cent sodium chloride, or 0.3 per cent beef extract, or peptone (a brand which was less satisfactory for supporting growth was tested for inhibition). The description given in Bergey's *Manual* of *Protaminobacter alboflavum* d would appear to permit a relationship, but a comparative study of bacteria of this type was not undertaken. It seems preferable, therefore, not to suggest a generic or species name for the A-splitting organism at the present time.

ACTION ON GROUP A SUBSTANCES

As already stated, the organism decomposes the group specific A substances from different sources, as human A saliva, horse saliva, and pig stomach (a material available in commercial pepsin and crude gastric mucin). The A preparations from pepsin and mucin have been used chiefly. During decomposition of the substances acid is formed (no gas is detectable by the common methods), and if the reaction is suitably readjusted to pH 6.5–7.0 at intervals during the period of incubation the organism will destroy the activity of 2 per cent solutions of the A preparations in the mineral base; this capacity for attacking the responsible serological structure is greater than that of *Saccharobacterium ovale* and exceeds by far the destructive activity of the *Myxococcus*. The action of the bacterium appears to lead to a thorough destruction, not for instance a mere splitting off of acetyl groups, since only traces of substances of polysaccharide nature were recovered following the action of the organism. No exoenzyme has been demonstrated. The bacterium did not attack the specific polysaccharides of *Pneumococcus* types I[6] or II[7], whereas *Pneumococcus* II polysaccharide is utilized by some other bacteria known to break down A substance, the *Myxococcus* of Morgan and *Saccharobacterium ovale* of Sickles and Shaw. In conclusion, the hope may be voiced that by means of such biological degradations of serologically active complex materials, information on the structure of these substances eventually may be yielded.

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[6] The preparation (SSS, prepared by the older methods) was kindly furnished by Dr. Harry Sobotka.

[7] This material was supplied through the courtesy of Dr. W. F. Goebel.

SUMMARY

The description is given of a bacterium having unusual characters and capable of destroying group A substances, that is, substances, presumably of polysaccharide nature, reacting with immune sera obtained by injection of human blood of group A. The organism does not grow on most common media, but can be cultivated in the presence of A substance.

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RELATIONSHIPS OF COLIFORM ORGANISMS

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Received for publication March 30, 1938

The generally accepted practice in classifying members of the coliform group is to designate as *Escherichia* those strains which produce indol but do not form acetyl-methyl-carbinol and are incapable of utilizing citrate as the sole source of carbon. Parr (1936) has suggested that this group of reactions be termed "IMViC." Thus *Escherichia* is IMViC $++--$. Strains reacting in the inverse fashion (IMViC $--++$) are designated as *Aerobacter*. A group with reactions resembling in part *Escherichia*, in part *Aerobacter* (IMViC $-+-+$) has been termed by some investigators "*Citrobacter*" while others prefer to include such organisms in the more general category of "intermediates."

With the standard IMViC differential tests there are 16 possible combinations of reactions. Moreover, if to these is added the ability to attack cellobiose (Koser, 1926) the number of possibilities is doubled, and if the production of acid or of acid and gas in the latter medium is taken into consideration there are theoretically 48 different types that could be obtained.

In an attempt to obtain as many of these types as possible and to study the frequency of their occurrence, some 5200 coliform cultures were isolated from human and bovine feces, dust of cow barns, and milk. Diluted fecal material was plated in eosin methylene-blue agar. The same procedure was used for milk except that in addition to eosin methylene-blue agar desoxycholate-lactose agar and "Bacto" crystal-violet red bile agar were also used. Eosin methylene-blue plates were exposed to the dust of cow barns and to material scraped from cow hides. All plates were incubated for 24 hours at 37°C. Typical coliform colonies were transferred from these plates to lactose broth. If acid and 20 per cent or more gas were produced, the broth was streaked on eosin methylene-blue agar and a well isolated colony selected for study. Indol, methyl red and Voges-Proskauer broths (Difco dehydrated), Simmons's citrate agar (Difco dehydrated), and cellobiose broths were inoculated with these cultures and incubated at 37°C. After 24 hours incubation the

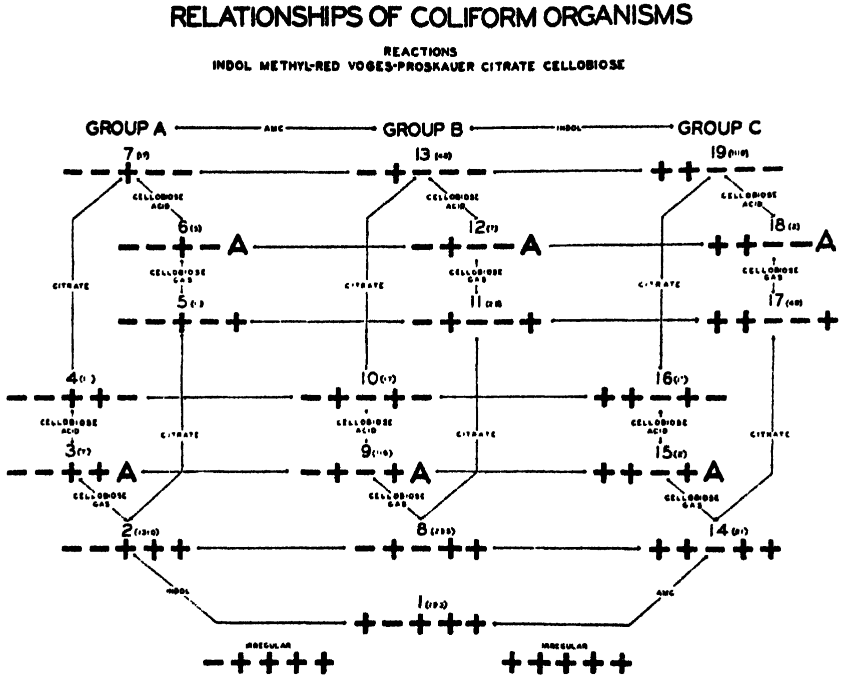

FIG. 1

indol broth was tested with Kovacs's reagent and the V-P broth with Levine's reagent. The M-R test was made with the standard reagent after 48 hours incubation. Reactions on the citrate agar and in the cellobiose broth were recorded daily for 5 days.

Of 4608 coliform cultures isolated and confirmed 3247 were grouped according to their reactions in the differential mediums. These cultures fall in orderly fashion into 3 groups of 6 types each, one type intermediate between 2 of the 3 groups, and 2 irregular types or a total of only 21 out of the 48 possible combinations. Figure 1 correlates the different types according to their biochemical activities. The numbers in parentheses, except where noted, represent the number of cultures giving the reactions characteristic of the type on the first test in the differential mediums. When new types were encountered, a number of cultures were plated on eosin methylene-blue agar and tested in the differential mediums 3 successive times. If the cultures were constant in all their reactions, they were placed in stock. Such cultures were studied at intervals over a period of from 3 to 9 months after isolation for changes in their biochemical reactions.

IRREGULAR TYPES

Early in the work a wide variety of color reactions was obtained in the M-R and V-P tests. To observe these reactions more closely the M-R V-P medium was pipetted in 4 ml. amounts into tubes selected for size and color of glass. In the test 0.1 ml. of the methyl red solution was used. Rather than to attempt to determine the pH accurately with so large a number of cultures 5 degrees of reaction were used in recording the results. The tubes were shaken and recorded as + when red, O^+ when intermediate between red and orange, O when orange, O^- when intermediate between orange and yellow, and − when yellow. In the V-P test 4 ml. of the reagent were used. The tubes were shaken, the reactions noted after 4 hours, and finally recorded after 12 hours at 37°C. as: strong, moderate, weak, trace, or negative according to the intensity of color.

Approximately 9 per cent of all cultures studied were positive to some degree in both the M-R and V-P tests. A majority of these cultures gave a moderate to strong V-P test with an M-R test varying from O^+ to O^-. Others were strongly positive in the M-R test with a weak, trace, or questionable reaction in the V-P test. Only 10 cultures, all isolated from one sample of milk, were strongly positive in both tests. When the M-R V-P medium was inoculated with these 10 cultures and tested daily for 2 weeks the M-R tests were + for from 5 to 7 days, then changed to O^+ or O^- but did not become really negative at any time. The V-P reaction under these conditions was consistently positive. When plated on eosin methylene-blue agar and incubated at 37°C. such cultures produced small, discrete, *Aerobacter*-like colonies. On subsequent incubation at room temperature for 24 to 48 hours the colonies developed to the large, raised, moist, confluent colonies characteristic in every respect of typical *Aerobacter*. Colonies from these plates or transplants from the parent culture transferred to the M-R V-P medium and incubated at room temperature were M-R negative and V-P positive in 24 to 48 hours. Growth in this medium was more luxuriant at room temperature than at 37°C.

Cultures giving a O^+ to O^- M-R reaction and a strong to moderate V-P reaction produced large and small colonies on eosin methylene-blue agar at 37°C. Large colonies gave a O^- to − M-R reaction at 37°C. and when replated produced both large and small colonies, the former predominating. Small colonies gave a + to O^+ M-R reaction and when replated produced both large and small colonies, the latter predominating. Both large and small colonies produced only large colonies at room temperature. Both large and small colonies when incubated at room temperature produced a negative M-R reaction over a 2 to 5 day period and a V-P reaction which might be strongly positive on the first day and weakly positive on the fifth day. Suitable experiments show that such cultures can utilize acetyl-methyl-carbinol and that the destruction is more rapid at room temperature. None of the 3247 cultures which produced both acid and gas in lactose, however, was negative in both the methyl-red and Voges-Proskauer tests.

Some cultures were isolated that produced a strongly positive M-R reaction and a weak or trace V-P test. These cultures produced typical intermediate or *Escherichia*-like colonies on eosin methylene-blue agar at 37°C. or room temperature, and the biochemical reactions were identical at both temperatures. Such cultures lost their ability to produce even a trace of acetyl-methyl-carbinol after from 3 to 9 serial platings or transplants. In these cultures a temperature effect does not seem to be involved and a characteristic is lost. Attempts to build up this character by selection within such a culture failed.

A further characteristic of the irregular types—and also of type 1—is the low volume (20–50 per cent) of gas produced in fermentations. While the volume of gas produced by coliform organisms is admittedly a variable factor, nevertheless we were frequently able to predict that a culture with the colonial characteristics of an *Aerobacter* but with a low gas volume would have the biochemical reactions of an irregular or of a type 1.

Type 1

The colonial characteristics of type 1 are those of *Aerobacter* except that the tendency toward confluence is not marked. Over 90 per cent of these cultures produced a low gas volume. No motile form was found, although all other types with a significant number of cultures showed both motile and non-motile forms. (The fact that motility is not included as a characteristic in figure 1 does not imply that we attach no significance to motility within a type.)

To test the constancy of the biochemical reactions, a varying number of cultures of the different types, whose biochemical reactions had remained unchanged after 3 successive platings and tests in the differential mediums, were transplanted serially on agar and tested in the differential mediums after each transplant. After 3 or 4 transplants the number of cultures being studied was usually reduced. For example: 22 cultures of type 1 were transplanted from agar to agar 3 times and their biochemical reactions tested after each transplant. Since no changes in biochemical reactions occurred, the number of cultures under observation was reduced to 12 for the next 2 transplants and tests, with a subsequent reduction to 5 for the next course of 3 transplants and tests. Thus, 22 cultures of type 1 showed no change in their biochemical characteristics after from 3 to 8 transplants over a period of from 1 to 6 months after isolation.

Groups A, B, and C

It will be seen (fig. 1) that the 3 major groups are aligned on the basis of their reactions in the indol and M-R V-P mediums.

Within the individual groups the biochemically most active types are at the bottom of the grouping with characteristics lost in ascending order.

Most of the cultures in groups A and B were isolated from sources other than feces. Of the 1310 cultures of type 2 only 39 came from feces, while no cultures of types 3–7 were isolated from this source. Of the 471 cultures in group B, 29 came from feces and the remainder from milk, dust, and hides. All 28 cultures of type 11 were isolated from milk. In group C, types 14 and 17 were isolated from all sources except cow feces and type 19 from all sources. The number of cultures in types 15 and 18 are too small to be significant as to source.

Type 9 is the only type in any group in which the number of cultures isolated is comparable to the number of cultures of the basic type of the group. An analysis of our data shows the following fact which may or may not be significant. During the period from the first of May to the middle of June there were isolated from all sources 182 cultures of type 8 and 11 of type 9. From the middle of June to the middle of August 23 cultures of type 8 and 96 cultures of type 9 were isolated, and from the middle of August to the first of October 50 cultures of type 8 and 9 of type 9 were isolated. The total number of coliform cultures isolated in the first period was greater than in the second period, while the total number isolated in the second period was greater than in the third period.

There are certain relationships in colonial form among the groups and types which have been observed. Thus, in group A, cultures of type 2 were generally of the large, moist, confluent sort typical of *Aerobacter*. Other types (except type 7) resembled *Aerobacter* in general although the colonies appeared to be more discrete and somewhat smaller in size. Type 7 produced a discrete, bluish, raised colony 3 to 5 mm. in diameter with a slight metallic luster. Colonies of group B were for the most part indistinguishable from *Escherichia* colonies except for types 8 and 9, many of which produced small, somewhat rough, dry colonies with an intense metallic luster. Colonies of group C were typical *Escherichia*, except for type 14 which were larger than ordinary and had a tendency toward confluence although they still retained a metallic luster.

The only type consistently producing a high gas volume (50–100 per cent) is type 2. All other types, even those in group A, produce less than 50 per cent gas. Indeed, type 7 never produced more than 20 per cent gas in 48 hours.

Study of the individual types during the course of successive transplants over a period of months indicated that certain strains were undergoing changes in their biochemical activities. These "shifts" in type are indicated in table 2. Certain salient features are immediately evident. Thus, in groups A and B, the shifts, with a single exception, involve the acquisition of biochemical activity. It is possibly significant that shifts are confined within the group with 2 exceptions, in which cultures of types 8 and 9 shifted to types 14 and 15 respectively, a shift from group B to group C. In this case, however, the shift from type 8 to type 14 is a shift in basic types and the cultures of type 8 involved were in a state of flux having just shifted from type 11 to type 8. Group C is peculiar in that it shows shifts to atypical forms (7 instances) more frequently than other groups. Shifts are not always in the direction of acquisition of characteristics as in groups A and B. There are 2 shifts, type 14 to 15 and type 18 to

TABLE 1

Types of coliform organisms isolated from various sources

SOURCE	NUMBER OF CULTURES TESTED	TYPES																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Milk	1358	106	690	0	0	10	4	17	200	67	12	28	0	7	28	0	0	35	0	154
Human feces	278	3	39	0	0	0	0	0	5	5	0	0	0	2	4	2	0	3	0	215
Cow feces	383	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	366
Hides	528	38	258	4	0	1	0	0	23	4	0	0	3	5	5	0	0	3	2	182
Dust	700	46	323	3	0	0	1	0	27	40	5	0	4	17	24	0	0	8	0	202
Total	3247	193	1310	7	0	11	5	17	255	116	17	28	7	48	61	2	0	49	2	1119

19, which involve losses in ability to attack cellobiose. Changes in utilization of citrate do proceed in the direction of acquisition. A further observation on group C which was not sufficiently well defined to warrant inclusion in the table involves a tendency of 8 non-motile cultures of type 14 to acquire an ability to produce acetyl-methyl-carbinol to some degree in from 2 to 5 transplants. This tendency to shift to type 1 is all the more interesting when

TABLE 2

Shifts in reactions of coliform cultures

ORIGINAL TYPE	SHIFT TO TYPE	NUMBER OF CULTURES STUDIED	NUMBER OF CULTURES SHIFTING	NUMBER OF TRANS-PLANTS TO PRODUCE SHIFT	CHANGE IN REACTION
1		22	0		
A 2	1	25	2	3-5	+ Indol
3	2	2	1	9	+ Gas cellobiose
4		1	0		
5	2	8	2	4	+ Citrate
6		3	0		
7	4	11	7	4 6	+ Acid cellobiose
7	6	1	1*		+ Citrate
B 8		10	0		
8	14	13†	2	4	+ Indol
9		6	0		
9	15	1‡	1*		+ Indol
10	9	10	1	6	+ Acid cellobiose
11	8	13	13	2-5	+ Citrate
12	13	2	1	4	− Acid cellobiose
13		5	0		
C 14	15	8	2	4	− Gas cellobiose
15	Atypical	2	2	2-3	− Gas lactose
16		1*	0		
17	14	13	2	2-6	+ Citrate
17	Atypical	13	5	2-6	− Gas lactose
18	19	2	2	1	− Acid cellobiose
19	16	19	3	2-4	+ Citrate

* Strain obtained by colony selection.

† Secondary shift following Type 11–Type 8 shift.

‡ Strain obtained from Type 10–Type 9 shift followed by colony selection.

it is noted that these 8 cultures are non-motile. It will be recalled that type 1 cultures are uniformly non-motile.

As is noted in table 2, not all of the shifts were obtained by the method of serial transplantation. Type 4 was never isolated from the specified sources. A culture of type 7 was plated on eosin methylene-blue agar and over 100 individual colonies fished and tested in the differential mediums. One variant was found that grew on citrate in five days. This strain was serologically identical with the parent strain. A similar result was reported by Minkewitsch and associates (1936). A culture of type 10 which acquired the ability to ferment cellobiose after 6 serial transplants (a shift to type 9) was plated on eosin methylene-blue agar in which the lactose had been replaced by cellobiose. Both black and white colonies were obtained. Black colonies produced acid in cellobiose in 24 hours and white colonies in 4 to 5 days. One of the colonies selected gave a weakly positive indol reaction after 2 transplants. On subsequent transplantation this culture increased in ability to produce indol, after which the characteristics remained constant.

Atypical coliform cultures

Approximately 600 cultures fished as typical *Aerobacter*, intermediate, or *Escherichia* colonies from dust, hides, and milk plates failed to confirm in lactose broth. Some of these cultures formed a bubble to 10 per cent gas in 4 to 6 days while others failed to produce any gas in 14 days. Such cultures when plated on eosin methylene-blue agar and incubated at 37°C. developed colonies which showed marked variation in size, ranging from those typical for a certain type to colonies barely visible. When left at room temperature, all the colonies on a single plate became comparable in size to those typical of the particular type.

Ninety-three of these cultures were selected for further study. They were replated, the differential mediums inoculated, incubated at 37°C. and at room temperature and tested after 36, 60, and 96 hours incubation. The reactions of representative cultures are shown in table 3.

Some cultures which produced no gas in lactose, were weakly

TABLE 3

Reactions of some atypical cultures

CULTURE NUMBER	TIME	37°C.						ROOM TEMPERATURE					
		Lactose	Indol	M-R	V-P	Citrate	Cellobiose	Lactose	Indol	M-R	V-P	Citrate	Cellobiose
	hours												
16 A	36	A	–	0^{+}	–	–	A	20	–	+	–	$+^{m}$	30
	60	A	–	0^{+}	–	–	B	20	–	+	–	$+^{s}$	30
	96	B	–	0^{+}	–	–	B	20	–	+	–	$+^{s}$	30
80 A	36	A	–	0^{-}	–	–	–	20	–	+	–	$+^{s}$	10
	60	A	–	0^{+}	–	–	A	20	–	+	–	$+^{s}$	20
	96	A	–	0^{+}	–	–	A	30	–	+	–	$+^{s}$	30
295 A	36	A	–	0^{-}	–	–	A	40	–	–	$+^{m}$	+	20
	60	A	–	0^{-}	–	–	A	60	–	–	$+^{s}$	$+^{s}$	30
	96	B	–	0^{-}	$+^{w}$	$+^{w}$	B	60	–	–	$+^{s}$	$+^{s}$	40
261 A	36	A	–	0^{+}	$+^{tr}$	–	–	30	–	–	$+^{s}$	+	30
	60	A	–	0^{-}	$+^{m}$	–	–	50	–	–	$+^{m}$	$+^{s}$	30
	96	A	–	0^{-}	$+^{w}$	–	A	80	–	–	$+^{m}$	$+^{s}$	50
366 A	36	A	–	0^{+}	–	–	–	20	–	+	–	$+^{s}$	30
	60	A	–	0^{+}	–	–	A	20	–	+	–	$+^{s}$	30
	96	A	–	0^{+}	–	–	A	20	–	+	–	$+^{s}$	30
457 A	36	A	+	0^{+}	–	–	B	20	+	0^{-}	$+^{m}$	+	20
	60	A	+	0	–	–	B	30	+	0^{-}	$+^{s}$	+	30
	96	A	+	0	–	$+^{w}$	10	30	+	–	$+^{m}$	$+^{s}$	30
499 A	36	–	–	0^{+}	–	–	–	20	–	0^{+}	–	+	20
	60	A	–	0^{-}	–	–	–	20	–	+	–	$+^{s}$	30
	96	A	–	0^{+}	–	–	A	20	–	+	–	$+^{s}$	30
628 A	36	B	+	+	$+^{w}$	–	10	20	+	–	$+^{m}$	$+^{s}$	40
	60	20	+	0^{+}	$+^{m}$	$+^{w}$	40	20	+	–	$+^{m}$	$+^{s}$	40
	96	20	+	0^{-}	$+^{m}$	$+^{m}$	40	20	+	–	$+^{w}$	$+^{s}$	40

$+^{tr}$ = trace.
$+^{w}$ = weak.
$+^{m}$ = moderate.
$+^{s}$ = strong.
A = Acid in carbohydrates.
B = Bubble of gas.
Numbers in carbohydrate columns indicate per cent of gas formed.

positive in the V-P test, and failed to grow on citrate at 37°C., were typical type 2 at room temperature. Others were V-P positive and citrate negative at 37°C. and positive in both reactions at room temperature. One culture (261A) which produced only acid in cellobiose at 37°C. and this only after 96 hours, produced acid and 50 per cent gas in this medium after a similar period at room temperature. The production of indol appears to be the same at both temperatures.

Of the 93 atypical cultures studied 53 produced acid and gas at room temperature. Thirty of these possessed the biochemical characteristics of type 2, 21 of type 8, and 2 of type 1. The remaining 40 cultures failed to produce gas at room temperature. Some of these were indol and M-R negative, strongly positive in the V-P and citrate mediums, produced acid in cellobiose and subsequently developed a red pigment (*Serratia*). Six cultures, all from milk, had the reactions of type 15 (++−+A) and one the reactions of type 19 (++−−−). No atypical culture producing indol which produced gas only at room temperature was obtained on primary isolation.

Several cultures isolated as atypical types 2 and 8 were found to produce gas in lactose at 37°C. after being in stock without transplanting for from 2 to 3 months. Typical type 2 cultures occasionally lost their ability to produce gas at 37°C. so that shifts from atypical to typical and from typical to atypical occur not infrequently.

Plant "coliform" cultures

In view of the source of all our atypical cultures—milk, dust, and hides—their biochemical reactions and particularly the inability of some cultures to produce gas from lactose at 37°C., a possible relationship to the organisms in the tribe Erwinieae is obvious. Accordingly eosin methylene-blue plates were streaked with decayed portions of a number of fruits and vegetables. After 48 hours at room temperature 200 coliform colonies were transferred to lactose broth and agar slants. Forty-two cultures, because of differences in colonial characteristics and reactions in lactose broth, were selected for further study.

The cultures were replated, transferred to lactose broth, and incubated at 37°C. and at room temperature. Thirteen cultures produced acid and gas at both temperatures, 24 produced gas at room temperature only, and 5 produced acid but no gas at either temperature. The reactions of these cultures in the differential mediums were determined at room temperature. Of the cultures producing gas, 6 were type 1, 21 type 2, 6 type 8, 1 type 9, and 3 type 14. Three cultures not producing gas at either temperature were $++--$A and 2 were $-+--$A.

It was hoped that the plant coliform cultures would, in most respects, resemble or be identical with the atypical cultures. There were certain differences apparent when the plant cultures were first isolated, but these became less marked on further cultivation under laboratory conditions. While a small percentage of typical and atypical cultures produced strong odors, the majority of the plant cultures did so when first isolated. The odor disappeared rapidly when the cultures were maintained under laboratory conditions. In lactose broth the plant cultures grew much more luxuriantly with a tendency toward ropiness which disappeared on subculturing. In the majority of atypical cultures the V-P reaction was moderate to strong. The plant cultures on primary isolation gave a trace to weak reaction if they were positive at all but the intensity of the reactions increased to moderate to strong over a period of 3 months. One hundred per cent of the plant cultures and all of the atypical cultures but one produced either acid or acid and gas from cellobiose. It is possible that the differences noted between the plant cultures and the atypicals may be due to differences in natural environment since the strains tend to become more alike when cultivated under the same conditions.

Serological investigations now in progress seem to show an antigenic relationship between the plant, atypical, and typical coliform organisms.

DISCUSSION

We realize that certain terms and procedures used in this paper require explanation. There may be some objection to selecting definite time limits (24 hours for indol and V-P tests, 48 hours for the M-R test, and 5 days for the reaction on citrate and cellobiose) in recording the reactions. We are quite in agreement with Gray (1932), Ruchhoft and associates (1931), and others in their observations that the time element is extremely variable. This is especially true in the case of the M-R reaction, where, had we allowed 5 days incubation, few irregulars would have been encountered. Cultures on citrate might in some cases develop a positive reaction in 2 or 3 weeks whereas they were completely negative in 5 days. The same is true of the reaction in cellobiose. Nevertheless these cultures were recorded as negative. The setting of a definite limit is necessary in comparing the reactions of over 3000 cultures where more than 8000 sets of differential tests are involved, and the limits we have set have proved very satisfactory for the great majority of cultures.

In describing our experimental results we have had occasion to refer to "shifts" in type of certain of our cultures. Ruchhoft and associates (1931) stress the necessity of purifying cultures before assigning them a definite position in classification. We are quite in agreement with this point of view, and all of our cultures were treated in a manner wholly analogous to that of these workers in "purifying" their cultures. All strains were replated and tested 3 times for constancy of reaction. We prefer to use the term "stabilization" for this treatment of the cultures since "purification" implies contamination by a foreign species and we have worked with strains as pure as is possible to obtain them short of single cell isolation. "Stabilization" implies a reasonable constancy of reaction without excluding the possibility of variation under suitable conditions.

While all our cultures were uniform in colony type and constant in reaction under the conditions previously noted, there were two situations which were encountered in treating the cultures in which constancy of cultural characteristics was apparently lacking. When eosin methylene-blue plates were streaked from the original lactose broth we found occasionally two or more colony types on the plate. Such findings were undoubtedly due to mixed cultures in the sense in which Ruchhoft refers to them. The individual types were, however, constant in their reactions after stabilization. On the other hand we have described certain atypical cultures which produced apparent mixtures of colony types at 37°C. while producing uniform types at room temperature. In this instance the effect is obviously a temperature characteristic of the strain and does not, we believe, imply a contamination of the culture.

In the work reported here it seems hardly likely that the orderly manner in which shifts occur could be entirely fortuitous. Were the results due to contaminations we would anticipate that the shifts would in most cases be to totally dissimilar types rather than to those closely related to the parent strain. Ruchhoft in one instance describes the isolation of three colonies from one of his purified strains which varied markedly in the manner in which they produced acetyl-methyl-carbinol. Some of our observations, especially on the tendency of type 14 to shift to type 1, are similar in import to these findings. With the exceptions noted our observations on shifts in reactions have been made on whole cultures in serial transplants. Thus we have been observing the activity of the entire bacterial population and the changes appear to be gradual. The problem is being reinvestigated at present by the method of colony selection to determine if possible whether the changes in type are gradual or appear suddenly in individual colonies. Another point that we hope will be clarified by this method is the nature of our so-called "irregular" strains, especially those which are strongly positive in both the M-R and V-P tests. On theoretical grounds we would anticipate perfect, or nearly perfect, negative correlation between these two tests. It may be, as Ruchhoft suggests, that cultures of this type are mixtures even though they have been stabilized and seem to be constant in their reactions. Our strains have been constant in their reactions over a series of 3 to 12 transplants and the "shifted" strains have remained stable for from 3 to 5 transplants.

It has been suggested by a number of investigators that the irregularities in the M-R and V-P reactions are due to lack of sufficient incubation time before performing the tests. While this is probably true, there can be little doubt that there is marked variation in the rate of reaction within individual strains. Thus Koser (1924) describes 8 strains which were M-R positive, V-P negative immediately after isolation which gradually changed until after a month or more they were M-R negative, V-P positive. This, of course, was under standardized conditions of testing. A similar condition has been observed in some of our atypicals and, as previously noted, 8 strains of type 14 showed a tendency to shift in this manner.

These irregularities in the M-R V-P reactions have been most noticeable in the so-called "atypical" strains, which, it will be recalled, are strains which either fail to produce gas from lactose or produce gas at room temperature only. Parr (1934) has noted that cultures are frequently isolated from fresh feces which are lactose-deficient and comments that such strains can usually be "trained" back to normal. We have noticed a similar acquisition of the ability to produce gas from lactose in many cultures, notably the plant "coliform" cultures. The fact that the acquisition of a positive V-P reaction appears to correlate with the development of the ability to produce gas from lactose leads us to suggest a possible mechanism for the change.

A probable series of reactions resulting in the formation of acetyl-methyl-carbinol involves the decarboxylation of pyruvic acid with subsequent condensation of the resulting acetaldehyde. Since, in fermentations, pyruvic acid is thought to result from the dehydrogenation of methyl-glyoxal hydrate, the formation of the precursors of acetyl-methyl-carbinol is a result of the action of the gas-producing enzymes. Sizer (1938) has suggested that the situation may be analogous to the course of certain enzyme-catalyzed reactions in which the end-point may be reached by more than one pathway. The energies of activation of the enzymes involved may so vary with temperature that one series of reactions will predominate over one temperature range while a second series will come to dominance as the temperature changes. It may be that the failure of certain strains to produce gas from lactose at incubator temperatures may be due to the dominance of enzymes leading to acid end products. Such a concept, or course, involves the assumption that those strains which acquire the gas-producing ability over a series of transplants eventually lose the ability to produce enzymes of this type. Koser (1924) comments: "It seems probable . . . that the secondary or alkaline fermentation was 'speeded up.' The production of acetyl-methyl-carbinol was never apparent until this reversion had taken place."

As to the validity of the types presented in figure 1 there can be little doubt. Skinner and Brudnoy (1932) although they made no distinction between the production of acid and of acid and gas in cellobiose, have described types which correspond to all save types 3–7, and 11 and 12. Oeser (1937), who records the production of gas in cellobiose, has isolated all types save types 4–7 and types 15 and 18. Both authors report strains which we would group with our irregulars.

At present we are reluctant to stress too strongly the "evolutionary" aspects suggested by the grouping in figure 1 and by the observed shifts in type. A number of investigators have commented on evolutionary tendencies in the coliform group and their possible origin from more primitive forms. Winslow and associates (1919) give what might be termed a "metabolic gradient" of the colon-typhoid group based on fermentative capacities although the authors state: "It cannot, however, be maintained that this progression necessarily or even probably represents the exact line of evolutionary development." The close similarity in the metabolic activities of the genus *Serratia* and members of the genus *Aerobacter* is well known, and, as Pederson and Breed (1928) point out, non-pigmented strains of *Serratia* may easily be mistaken for *Aerobacter*. Frobisher (1937) refers to the *Serratia* group as "infra-aerogenes."

Certain of our recorded observations appear to suggest such possibilities. Considering the basic type in group A (type 2): cultures have been obtained which vary in gas production all the way from complete inability to produce gas at any temperature up through types which produce 100 per cent of gas at incubator temperature. The basic type of group B (type 8) shows similar relationships except that the maximum volume is less than 50 per cent.

The numerical relationships between the types in the different groups suggest that in their natural habitat the organisms may "evolve" into types 2, 8, and 19. The nature of the shifts observed tends to confirm this concept, although we are at a loss to explain the relative scarcity of type 1. It may be that such "evolutionary" activity, if it exists, proceeds from two directions with a tendency to stabilize at the basic types. Until more data are available we feel justified only in calling attention to the fact that changes in type seem to follow closely the scheme laid down in figure 1.

The results reported in this paper do, we believe, contribute in some degree to the question of classifying the members of the coliform group. Suggestions for the classification of this group range all the way from the creation of a separate genus, "*Citrobacter*," (Werkman and Gillen, 1932) to the elimination of all save the species *Escherichia communis* and *Aerobacter aerogenes*. In the middle ground Tittsler and Sandholzer (1935) deem it possible to allocate the intermediate types to the genus *Escherichia* with the distinction between *Escherichia* and *Aerobacter* based on the production of acetyl-methyl-carbinol. Carpenter and Fulton (1937) also stress the inadvisability of creating a separate *Citrobacter* genus. On the other hand the frequency with which intermediate types are encountered makes us feel that it would be unwise to regress too far in the direction of simplification. The importance of this group of organisms in the determination of the sanitary quality of water, milk, shellfish, and other food-stuffs makes it desirable that we retain a distinction between the various groups.

By grouping the members of the coliform section as indicated in figure 1 it is possible to place the great majority of types encountered with a minimum of effort and expense in one of three fundamental groups. This can be accomplished by the use of but two reactions, indol and Voges-Proskauer (or methyl-red). For more careful classification, citrate and cellobiose may be used, but in the majority of instances this would prove unnecessary and even confusing.

The subdivision of the coliform section into three major groups seemed most suitable in view of the numerical relationships of the various types and the direction of shifts in type. Statistical analysis of the data confirms this concept and serves to place it on a less empirical basis. Winslow and Rogers (1906) and Levine (1918) have applied correlation coefficients to the problem of classification. While distinction between species should be based on those reactions showing the least correlation, major subdivisions of a large group of organisms should be characterized by reactions as highly correlated as possible. The statistical treatment of our data indicates that the grouping given in figure 1 most nearly accomplishes this purpose. Tetrachloric coefficients of correlation (table 4) were computed for all the reaction pairs used in differentiating the members of the coliform group. The distinction between types within a given group is based on the reactions in citrate and cellobiose, which are the least correlated of the reaction pairs. Separation into three major groups on the basis of indol and V-P (or M-R) reactions serves the same end in that these reactions are thus perfectly correlated within the group whereas they would not be were the distinction made on the basis of V-P and citrate.

TABLE 4

Coefficients of correlation of reaction pairs in differential mediums

	INDOL	M-R	V-P	CITRATE	CELLOBIOSE
Indol		0.90	−0.90	−0.97	−0.98
M-R	0.90		−1.00	−0.98	−0.99
V-P	−0.90	−1.00		0.98	0.99
Citrate	−0.97	−0.98	0.98		1.00
Cellobiose	−0.98	−0.99	0.99	1.00	

We would suggest, then, that the members of the coliform group of organisms might be divided into three sections on the basis of their reactions in indol and Voges-Proskauer mediums. The sections might be termed *Aerobacter*, Intermediate, and *Escherichia*.

SUMMARY

1. Organisms of the coliform group (3247 strains) fall into 3 major groups of 6 closely related types each.

2. "Irregular" and "atypical" strains encountered may represent primitive forms of the coliform group.

3. It is suggested that the coliform group of organisms be divided into 3 sections: *Aerobacter*, Intermediate, and *Escherichia* on the basis of their indol and Voges-Proskauer reactions.

The authors wish to acknowledge assistance rendered by Mr. R. P. Elrod, Mr. R. C. Bitterlich, and Miss Carolyn E. Brown.

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COLIFORM ORGANISMS IN CERTIFIED MILK

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Received for publication March 30, 1938

While investigators agree that coliform organisms in milk are of little significance with respect to conditions of production, a number have suggested that colon counts on high quality milks may be of some value under certain conditions. Ayers and Clemmer (1918) and Sherman and Wing (1933) suggest that the presence of coliform organisms is significant only if the total bacterial count is less than 10,000, the milk is relatively fresh, and the holding temperature has been below 50°F. Certified milk is required to meet these conditions. The American Association of Medical Milk Commissions has approved a standard of less than 10 coliform organisms per milliliter, and has recommended the use of desoxycholate agar mediums (Leifson, 1935) for plating. Two types of desoxycholate agar mediums are available commercially. These differ primarily in their concentration of sodium desoxycholate. The higher concentration (0.1 per cent) is recommended for certified milk or when 1 ml. of whole milk is to be plated, while the medium with the lower concentration (0.05 per cent), so-called "desoxycholate-lactose agar" is to be used when diluted milk is plated.

To obtain a comparison of the two desoxycholate mediums 80 samples of certified milk were plated in both. In addition 40 samples of producers' raw, a relatively low quality milk, were carried through the same procedure. Sixty of the certified and all of the producers' raw milks were also plated in eosin-methylene blue agar. The results of the comparative tests are shown in table 1. For each kind of milk the average counts on desoxycholate agar were only about 62 per cent of those on desoxycholate-lactose agar, even though the certified milks were plated undiluted. Coliform counts on eosin methylene-blue agar were essentially the same as on desoxycholate-lactose agar. These comparative counts show that the medium containing the lower concentration of sodium desoxycholate is more satisfactory for the enumeration of coliform bacteria even in low count milks. The greater inhibition of non-coliform organisms by desoxycholate-lactose agar renders it superior to eosin methylene-blue, especially in routine examination of high count milk.

Weekly or semi-weekly samples of certified and producers' raw milks were plated, usually in desoxycholate-lactose agar. If coliform colonies developed they were carefully examined, representative numbers of the different kinds transferred to lactose broth, and the reactions noted after 24 hours. Such confirmatory tests were applied to 2252 coliform colonies from certified and producers' raw milks. Acid and gas were produced by 81.1 per cent of the cultures and acid only by 18.9 per cent. ("Atypical" cultures, producing only acid in lactose, will be discussed in a subsequent paper.) Of the 1826 cultures producing acid and gas, 1106 were plated on eosin methylene-blue agar.[1] Indol, methyl-red and Voges-Proskauer broths, Simmons's citrate agar, and cellobiose broth were inoculated from well-isolated colonies. Indol and Voges-Proskauer tests were made with Kovacs's (1928) reagent and Levine's (1934) modi-

TABLE 1

Comparison of plating mediums for coliform count

MILK	SAMPLES TESTED	COLIFORM PRESENT	AVERAGE COLIFORM COUNT ON		
			Desoxycholate agar	Desoxycholate lactose agar	Eosin methylene-blue agar
Certified	80	43	49	78	75*
Producer's raw	40	35	125,000	204,000	215,000

* Only 60 samples were plated on eosin methylene-blue agar.

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[1] Eosin methylene-blue agar: 12.5 grams proteose peptone, 200 mgm. eosin, 50 mgm. methylene blue, 10 grams lactose, 10 grams Bacto agar, 1000 ml. distilled water. On this modification of eosin methylene-blue coliform colonies can be differentiated easily from non-coliform colonies in from 12 to 15 hours.

fication of O'Meara's reagent, respectively, after 24 hours incubation at 37°C. Methyl-red tests were made after 48 hours incubation. Growth on citrate agar and the reaction in cellobiose broth were observed daily for 5 days.

During 6 months (April to September) 233 samples of certified milk from 5 farms were examined. The incidence of samples with coliform organisms is given in table 2. Cultures were recorded as *Escherichia*, intermediate, or *Aerobacter* according to the accepted reactions of the groups in the differential mediums.

TABLE 2

Incidence and sectional differentiation of coliform organisms from milk

MILK	SAMPLES EXAMINED	COLIFORM PRESENT	MORE THAN 10 COLI PER ML.	AVERAGE COLI PER ML WHEN PRESENT	NUMBER SAMPLES FROM WHICH COLI WERE ISOLATED	CULTURES STUDIED	ESCHERICHIA	INTERMEDIATE	AEROBACTER
		per cent	*per cent*				*per cent*	*per cent*	*per cent*
Certified farm B. . . .	30	63	3	4	15	103	13.5	10.8	75.7
Certified farm C.	60	98	78	97	23	229	0.0	47.2	52.8
Certified farm F	63	46	10	27	14	94	9.6	10.6	79.8
Certified farm W. . .	31	48	0	1	12	47	27.7	8.5	63.8
Certified farm Fe . . .	49	71	37	142	24	148	18.9	45.9	35.2
Total	233				88	621	10.3*	32.4*	57.3*
Producer's raw . . .	71	80	76	147,000	52	485	9.1	16.7	74.2

* Per cent of 621 cultures studied.

Three certified milk farms were selected for investigation as to possible sources of the coliform organisms. Direct isolation on eosin methylene-blue plates was used. Sterile plates were exposed to the dust of the barns and dairies and to material scraped from the sides, bellies, and udders of cows. Samples of fresh cow feces were collected and dilutions plated in eosin methylene-blue agar. Coliform colonies from the various sources were transferred to lactose broth, confirming cultures plated on eosin methylene-blue, and selected colonies studied as previously described. This procedure was repeated 3 times at each farm.

From the feces of 30 cows from the 3 herds, 383 coliform cultures were studied. One hundred per cent of these were *Escherichia* despite the fact that every colony with any *Aerobacter* characteristics was fished. It is obvious that only the *Escherichia* present in these milks could be due to the bacteria from cow feces. That *Escherichia* from this source should infect milk while the other types of coliform bacteria in the same milk come from entirely different sources seems quite improbable.

TABLE 3

Sectional grouping of coliform organisms from milk, stable dust, and cow hides

FARM	MATERIAL	COLONIES	ESCHERICHIA	INTERMEDIATE	AEROBACTER
			per cent	*per cent*	*per cent*
C	Milk	229	0.0	47.2	52.8
	Dust	320	18.5	12.8	68.7
	Hides	275	20.4	10.6	69.0
F	Milk	94	9.6	10.6	79.8
	Dust	189	81.5	5.8	12.7
	Hides	54	92.6	0.0	7.4
Fe	Milk	148	18.9	45.9	35.2
	Dust	278	14.7	27.7	57.6
	Hides	192	45.8	3.6	50.6

Farm "C" is particularly interesting in this respect since no member of the *Escherichia* section was isolated from its milk.

Table 3 shows the percentages of the 3 sections of coliform organisms found in the milk as compared with dust and hides. The results as given include examinations of the milks over 6 months, while the specimens from dust and hides were collected during 2 to 4 months. The diametrical opposition of the flora of the milk from farm "F" to that of the dust and hides is particularly striking, as is the fact that of the large number of cultures isolated from the milk of farm "C" none was of the *Escherichia* section. The differences in the case of farm "Fe" are less striking, but inasmuch as hand milking is practiced the chances of air contamination are greater. Considerable variation in the percentage distribution at the different farms was observed, but in no case was the distribution similar to that observed in the milks.

DISCUSSION

Numerous mediums, both liquid and solid, have been recommended for the isolation and enumeration of coliform bacteria in milk. The merits of a large number of these have been studied and discussed extensively by Bartram and Black (1936) and Yale (1937a). Granted a suitable medium the major objection to the use of liquid mediums is the large number of tubes which must be used if the determination is to be quantitative. On the other hand, both these authors have found that a considerable number of the colonies developing on solid mediums fail to confirm in lactose broth. In the present study 18.9 per cent of such strains were encountered. Yale (1937a) has called attention to the difficulty of classifying these organisms and assigns his strains variously to the genera *Achromobacter*, *Flavobacterium*, *Serratia*, and *Alcaligenes*. Oeser (1937) has noted the close relationship of many of these organisms to the genera *Erwinia*, *Phytomonas*, and *Klebsiella*. A subsequent paper will discuss the reactions of these "atypicals" and their biochemical and serological relationship to the coliform group.

There is extensive evidence that the coliform count in raw milk in general is of little sanitary significance. (Ayers and Clemmer, 1918; Bartram and Black, 1937b; Kline, 1935; Sherman and Wing, 1933; Yale et al., 1935, 1937b.) In pasteurized milk the presence of the group has an entirely different meaning. (Chilson et al., 1936, Yale, 1937a, b.) In certified milk, however, the conditions are such as led Ayers and Clemmer (1918) and Sherman and Wing (1933) to suggest that the coliform count may serve as a supplementary index of sanitary quality. If, as Bartram and Black (1937) suggest, the tendency to regard all coliform organisms as of possible fecal origin is valid, then the presence of any members of the group would indicate a highly undesirable condition. Parr (1936, 1937) has noted a "succession of forms" in stored feces where the *Escherichia* types found in fresh samples soon disappeared and were replaced by intermediates and members of the *Aerobacter* group. Moreover many of the *Aerobacter* types were "lactose degraded" and might correspond to the "atypical" strains which we have encountered. On the other hand, Tonney and Noble (1931) found no significant change in relative numbers of *Escherichia* and *Aerobacter* as they occur in fecal material, nor was there a material difference in the survival time of the types.

Kline (1935), on the basis of percentage distribution of coliform types in milk of different grades, feces, hay and grain, and pasture soil, concluded that reliance should be placed on the identification of *Escherichia* if an index of fecal pollution were desired. On a similar basis (table 3) we would incline to the same opinion. The milk flora by no means showed any correspondence to bovine fecal contamination since no members of the *Aerobacter* or intermediate sections were encountered in cows' feces, and it must be admitted that the findings in dust and hides do not warrant the assumption that they are the sole source of contamination. In view of the high incidence of members of the *Aerobacter* section in these milks the findings of Ayers and Clemmer (1918) suggest the probability of utensil contamination as the source of the coliform organisms encountered.

Too much stress should not be placed on the occurrence of members of the coliform group as a whole in certified milk. The extensive observations of other investigators quoted herein and our own findings render the presence of members of the *Aerobacter* and intermediate sections insignificant as an index of fecal contamination, yet a rigid insistence on adherence to the standards set for the coliform count would result in the condemnation of such milks as that of farm "C" which had an average coliform count of 97 over a period of 6 months (table 2) with no member of the *Escherichia* section encountered.

SUMMARY

Classification of coliform organisms isolated from certified milk showed considerable variation in proportions of *Aerobacter*, intermediate, and *Escherichia* types with a marked predominance of *Aerobacter* and intermediate forms. Since the three groups differ in their sanitary significance the total coliform count seems to be of doubtful value.

We wish to acknowledge a financial grant for the purchase of materials from the Association of Certified Milk Producers and from the Fair Oaks Certified Farm, Providence, R. I.

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A CINEMATOGRAPHIC ANALYSIS OF THE MOTION OF COLONIES OF B. ALVEI

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Received for publication April 14, 1938

At the 1937 meetings of the Society of American Bacteriologists, Smith and Clark reported (1938) on the motility of entire colonies of *Bacillus alvei* and other organisms. Being interested at the time in lapse-time motion picture studies of bacterial colonies, we obtained a culture through the agency of the American Type Culture Collection. The marking on this culture leads us to assume that it came originally from Smith.

The strain was found to be motile as described. Preparation for cinematographic study comprised seeding one point on the margin of a freshly poured plate of 2 per cent Proteose-Peptone, beef infusion agar (1.5 per cent agar) of pH 7.4. The exact amount of surface moisture was found to be highly critical, and several plates of varying degrees of dryness were always set up. These were incubated briefly until the plate showing rapid motion of discrete colonies across the fresh medium could be selected with the aid of a hand lens. This plate was then placed in the incubator (30°C.) of the photographic equipment and a record made of about two days' growth at 15 frames per hour. This resulted in a 3800 times magnification of speed when the film was projected at the normal rate. On one hundred feet of film we have recorded the action on 10 plates.

The development of a typical culture appears to fall into five general periods:

1. The first growth consists of minute colonies which move across the field at high speed in any direction. The path is rarely linear for any distance. Rather, it is usually a series of wide circling movements. The number of such active colonies increases rapidly until the second phase sets in.

2. The colonies now come to rest, not gradually, but very abruptly. Frequently the small stable colony so formed will have a residual motion of rotation. The abruptness of the halt eliminates any question of "running down," and I think it is to be explained by an occurrence which is frequently seen under the microscope, namely, a turning back of the head of the advancing colony so that it forms a sharp left hand loop which quickly progresses to form a spiral and then, as the spiral tightens, a compact colony which still possesses the rotary motion to which its linear motion has been transformed.

3. When nearly all the colonies have entered upon the second phase, the third begins. This is one of comparative rest with continued growth in size.

4. The fourth phase embraces a second active period in which colonies apparently at rest take on a slow rotary motion which accelerates and becomes more extensive as time goes on. This is photographically the most striking stage. As the rate increases, spiral arms are extended from many colonies so that they resemble spiral nebulae. Fragments are frequently detached to lead an independent existence and may depart entirely or continue to circle the parent as a satellite. Some colonies will detach a complete ring of growth which continues to rotate about the center at some distance from it. These changes resemble those caused by centrifugal force, but this is, of course, illusory.

5. In the last stage the culture "freezes" into a final stability. In contrast to the abrupt halt in the initial phase, this stoppage is gradual. The spirals and rings rotate more and more slowly until finally all motion is lost. This seems in general to be the result of overcrowding but may be in part due to loss of surface moisture.

This last point is borne out by an accident which took place in one series. During the run the temperature in the incubator fell for a short time with a resultant condensation of moisture on the glass cover of the petri dish. In viewing the film it was noted that, simultaneously the culture, which was in the early 4th stage, ceased its rotations. When the damage was repaired, with consequent rise in temperature and redistillation of the condensed water from the glass back to the surface of the now cooler agar, the motion was abruptly resumed. Since this motion does not appear to be impaired by room temperature as opposed to 30 degrees Centigrade, moisture would seem to be the controlling factor in this instance.

The timed nature of the lapse-time picture permits ready measurement and comparison of velocities on numbers of colonies. The following average values are typical:

Rotations (phase 4):	
Colonies 1-1.25 mm. in diameter	1.4 r.p.h.
Colonies 1.25–1.75 mm. in diameter	0.8 r.p.h.
Colonies 1.75–2.25 mm. in diameter	0.5 r.p.h.
Linear motions (phase 1):	
Colonies 0.2–0.5 mm. in diameter	14 mm./hr.

It may be interesting to compare the last value with stated velocities for single motile cells of other species.

Eberthella typhi	65 mm./hr.
Bacillus megatherium	27 mm./hr.
Bacillus alvei (colonies)	14 mm./hr.

When we consider the bulk of the *B. alvei* colony, as compared to that of a single cell, the value 14 appears to be quite respectable.

Another interesting observation is that, of the probably 200–300 rotating colonies shown in the film, only two have been detected whose motion is clockwise. The remainder are uniformly counterclockwise. This brings up again the controversy which has been raised at numerous times in the past over the reason for such directed rotation in the biological world.

To return finally to the paper by Smith and Clark, I must call attention to two points: first, the statement that (as distinguished from the motion of *Bacillus circulans*) "in the *B. alvei* type the colony moves forward like a bullet and may take any direction." It would appear evident from the motion picture studies that Smith's statement is true only for phase 1 and that in the later stages the most characteristic feature is a rapid and extensive rotation with no motion of translation. It is only fair to state that we did not have more than a vague suspicion of the existence of this rotary motion until the pictures were available. Second, while not in a position to suggest the true mechanism, I must question the conclusion that "the motility of the colonies is not a characteristic of any one species, but that it is the result of a strong motility of the individual cells combined with certain physical conditions of the medium." I doubt if this can be the entire answer, for, while we may grant that the motive power is derived from the motility of the single cell and that the condition of the surface is a highly critical factor, we still leave unexplained the unified action of the cells so that the colony moves as a whole rather than spreading in all directions as in the case with other highly motile strains. It would seem necessary to postulate some mechanism by means of which the individualists of the cell population are induced to submit to a regimentation.

It is my impression that colonial motility in *B. alvei* has been described only in Smith's report. It is difficult to understand why such an outstanding characteristic should have been overlooked. In an article by Muto (1904) there is described an organism with strikingly similar properties. The motions of this strain (*Bacillus helixoides*) are apparently identical with those described here for *B. alvei.* The question is then raised as to whether the strain in my possession is *B. alvei* or *B. helixoides* or whether, in last analysis, these two species, which according to reported descriptions are distinctly different in morphology and staining, are one and the same. The strain in my possession would, on cursory examination, answer best to Muto's description.

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COMPARATIVE STUDIES ON THE PURIFICATION OF TETANUS AND DIPHTHERIA TOXINS

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Received for publication April 20, 1938

Diphtheria toxin has been identified as a typical heat-coagulable protein (Eaton 1936 a, 1937; Pappenheimer 1937). If other toxins have chemical properties similar to diphtheria toxin, they might be isolated by similar methods.

Using a gelatin hydrolysate medium, Pappenheimer and Johnson (1936, 1937) have produced diphtheria toxin which may be obtained in a relatively pure state by simple fractionation with ammonium sulphate. However, strong tetanus toxin has not been produced on a simplified medium similar to that used by Pappenheimer and Johnson for diphtheria toxin. Consequently, attempts to isolate tetanus toxin must be confined, at present, to the separation of the toxin from the proteins, proteoses, and peptones of the usual complex media.

The method of purifying toxins by precipitation with alum and cadmium chloride, as previously described (Eaton 1936 a, b), has been simplified by omitting the precipitation with alum and by making certain other modifications. Diphtheria toxin made on the proteose-peptone medium of Wadsworth and Wheeler (1934) and tetanus toxin, made on a meat infusion medium, may be considerably purified without appreciable alteration by a single precipitation with cadmium chloride.

For the purpose of comparing the properties of diphtheria and tetanus toxins, the toxins must be made on similar media and purified by similar methods unless the complete purity of the final products can be proved. Differences in the properties of the two toxins necessitate the use of slightly different methods in their purification. For this reason the method applied to the purification of each toxin is described in enough detail to make clear the differences found.

PRODUCTION AND PURIFICATION OF DIPHTHERIA TOXIN

The crude toxin was produced by growing the Park-Williams no. 8 (5) strain of *Corynebacterium diphtheriae* on the proteose-peptone medium described by Wadsworth and Wheeler (1934). Details will be found in a previous paper (Eaton 1936 a). To the toxic filtrate are added sufficient sodium citrate to make the concentration 2 per cent and then $\frac{1}{5}$ volume of 5 per cent cadmium chloride solution. The pH should be about 7.0. The toxin solution should be stirred thoroughly during the addition of the cadmium chloride, and occasionally for an hour or two afterwards. The resulting precipitate is allowed to settle and the liquid decanted and filtered. The precipitate, which consists mostly of phosphates with some adsorbed impurities, is discarded. To the filtrate $\frac{1}{3}$ volume of 5 per cent cadmium chloride solution is added and the pH is adjusted to 6.0. A fine granular precipitate forms slowly. After the preparation has stood in the refrigerator overnight, the precipitate containing the toxin is collected and washed with water. The washed precipitate is eluted with 2 per cent sodium phosphate at pH 7.8 in the refrigerator for 24 hours. The resulting partially purified toxin is further purified by fractionation with ammonium sulphate and dialysis (Eaton 1936 a; Pappenheimer 1937). Separation of toxin from bacterial protein may be accomplished by the method already described (Eaton 1937).

PRODUCTION AND PURIFICATION OF TETANUS TOXIN

Tetanus toxin was produced from a strain of *Clostridium tetani* isolated in this laboratory. In previous purification experiments (Eaton 1936 b) a strain of *C. tetani* obtained from the New York State Laboratories was used for the production of toxin. The cultures were grown in two-liter Erlenmeyer flasks filled almost to the neck and sealed with vaseline. The toxin was filtered after incubating the cultures for two weeks. The medium consisted of the ordinary veal infusion broth containing 2 per cent of Bacto-peptone and 1 per cent of glucose.

The filtrate is treated with $\frac{1}{20}$ volume of 20 per cent calcium chloride solution and filtered to remove phosphates. To the filtrate adjusted to pH 6.7 to 7.0 is added sodium chloride to a concentration of 5 per cent and then $\frac{1}{2}$ volume of 5 per cent cadmium chloride solution. If no precipitate forms, the pH of the solution is raised by the addition of N/1 NaOH until a slight turbidity appears. If the precipitate is very heavy, the pH should be lowered (see next section). After standing overnight the precipitate is collected, washed, and eluted as described for diphtheria toxin.

The eluted toxin is precipitated by ammonium sulphate in the fraction between 0.4 and 0.6 saturation. The ammonium sulphate may be removed from the redissolved precipitate by dialysis in a cellophane bag. In the attempts to further purify tetanus toxin by fractionation with ammonium sulphate and dialysis much of the toxin was lost. This is apparently due to the instability of the purified toxin.

For the determination of the M.L.D. of crude and purified tetanus toxin, broth was used for a diluent because the toxicity could not be measured satisfactorily by diluting the toxin in saline. Halter (1936) and others have observed that crude tetanus toxin diluted in saline is less stable than when it is diluted in broth. This is probably attributable to a protective effect of the broth, not to the activation of the toxin by anything in the broth. Bronfenbrenner (1924) has made similar observations with botulinus toxin.

THE EFFECT OF SODIUM CITRATE, SODIUM CHLORIDE, AND pH ON PURIFICATION OF TOXINS BY PRECIPITATION WITH CADMIUM CHLORIDE

Cadmium chloride alone added to crude toxin or the sterile culture medium precipitates not only proteins but also proteoses and possibly other polypeptides. The amount and character of the material precipitated depends on the pH. In a solution containing 2 per cent sodium citrate or 5 per cent sodium chloride, the compounds formed between cadmium chloride and the nitrogenous substances are more soluble, and an excess of the reagent is required to produce precipitation. Under these conditions the solubility of the nitrogenous impurities is relatively greater than the solubility of the toxic fraction. The character of the material precipitated depends on the amount and kind of salt present (sodium chloride or sodium citrate), the concentration of cadmium chloride, and the pH. In preliminary experiments tetanus toxin was precipitated by cadmium chloride in the presence of 2 per cent sodium citrate. Later, it was found that better yields of purer toxin were obtained by using 5 per cent sodium chloride in place of the citrate.

With both tetanus and diphtheria toxins the higher the pH the more precipitate is formed, and the more impure is the resulting preparation. Maximum purification is obtained by precipitating the toxin at the lowest pH consistent with a reasonable yield of pure toxin. For diphtheria toxin the optimum pH is near 6.0. For tetanus toxin the optimum pH is higher, generally between 6.3 and 7.0. Since this varies with different lots of toxin, the pH of precipitation with maximum yield and purification should be determined in small samples before precipitating the bulk of the toxin.

RESULTS OF THE PURIFICATION PROCESS

Diphtheria and tetanus toxins were purified to about the same degree by the methods just described, and the yields of toxin were similar in each case (table 1). In terms of nitrogen per M.L.D., diphtheria toxin purified by precipitation with cadmium chloride is not as pure as the best preparations previously obtained by more complex procedures from crude toxins prepared on the same medium, but its purity compares favorably with that of toxin purified by any procedure involving only one precipitation. By fractionation with ammonium sulphate and dialysis, impurities in the form of inactive protein and proteoses are separated from the toxin and the resulting preparation contains only slightly more nitrogen per M.L.D. than the purest diphtheria toxin so far obtained. The tetanus toxin purified by cadmium precipitation is at least twice as active as the preparation previously obtained by a more complex procedure (Eaton 1936 b). As indicated in the last two lines of table 1, fractionation of the purified tetanus toxin with ammonium sulphate and dialysis resulted in a marked reduction of the yield, and little further purification was accomplished. Part of the loss of toxin occurred during the precipitation with ammonium sulphate, and 50 to 75 per cent was lost during dialysis in cellophane.

TABLE 1

Results of the purification of tetanus and diphtheria toxins

TOXIN	PURIFICATION PROCEDURE	N PER M.L.D.	TEST ANIMAL	PURIFICATION FACTOR	YIELD
		mgm.			*per cent*
Diphtheria	Crude	0.00520	Guinea pig		
Diphtheria	cd	0.000100	Guinea pig	52	75
Diphtheria	cd	0.000044	Guinea pig	120	65
Diphtheria	cd., A.S.	0.000028	Guinea pig	186	60
Tetanus	Crude	0.000250	Mouse		
Tetanus, Lot A	cd	0.0000057	Mouse	44	80
Tetanus, Lot B	cd	0.0000020	Mouse	125	90
Tetanus, Lot A	cd., A.S.	0.0000041	Mouse	61	10
Tetanus, Lot B	cd., A.S.	0.0000018	Mouse	138	22

Purification procedure: cd, precipitation with cadmium chloride as described in text; A.S., fractionation with ammonium sulphate.

Nitrogen determined by the micro-Kjeldahl method of Pregl.

$$\text{Purification factor} = \frac{\text{Mgm. N/M.L.D. in crude toxin}}{\text{Mgm. N/M.L.D. in purified toxin}}.$$

$$\text{Yield} = \frac{\text{Total M.L.D. in purified toxin}}{\text{Total M.L.D. in crude toxin}}.$$

THE COMPOSITION OF TETANUS TOXIN COMPARED WITH DIPHTHERIA TOXIN

The results presented in table 2 indicate that tetanus toxin purified to the same degree as diphtheria toxin by a similar method contains relatively little protein. Although practically all the nitrogen in the purest diphtheria toxin is precipitable by trichloracetic acid, only 8 to 18 per cent of the nitrogen in the preparations of purified tetanus toxin is precipitable as protein. If the 8 per cent of protein in one of the preparations were all toxin, the lethal dose of this protein for mice would be 0.0000000008 gram, an almost unbelievably small figure. By fractionation with ammonium sulphate and dialysis the toxicity and non-protein nitrogen are reduced and the protein nitrogen is increased to 35 per cent. This does not necessarily mean that the toxin is associated with the non-protein fraction which diffuses through the membrane. It is possible that the activity of the protein is destroyed without appreciably affecting its chemical properties. Sixty to 80 per cent of the nitrogenous material in the preparations of purified tetanus toxin is precipitable by tannic acid. This indicates that a large proportion of the nitrogen is in proteose or polypeptide form. The remainder of the nitrogen (20 to 40 per cent) is in substances of unknown nature.

TABLE 2

Total nitrogen and protein nitrogen in purified preparations of diphtheria and tetanus toxins

TOXIN	PURIFICATION PROCECURE	M.L.D. PER CC.	TOTAL N PER CC.	PROTEIN N PER CC.	PROTEIN N IN TOTAL
			mgm.	*mgm.*	*per cent*
Tetanus, Lot C...	cd	35,000	0.095	0.018	18
Tetanus, Lot B...	cd	40,000	0.078	0.005	8
Tetanus, Lot A.... .	cd	40,000	0.235	0.014	10
Tetanus, Lot A... . ..	cd., A.S.	8,000	0.033	0.011	35
Diphtheria	cd., A.S.	6,000	0.157	0.118	75*

Protein nitrogen is the nitrogen in the precipitate produced by 5 per cent trichloracetic acid.

* In more highly purified preparations of diphtheria toxin the protein nitrogen approaches 100 per cent.

In the preparations of purified tetanus toxin obtained by a different method from a different strain of *C. tetani* (Eaton 1936 b) 25 to 50 per cent of the nitrogenous material was precipitated by trichloracetic acid as protein. Those preparations were less pure than the ones described in the present paper, and it is possible that they contained more inactive protein.

THE EFFECT OF ACID ON TETANUS AND DIPHTHERIA TOXINS

Like many proteins, diphtheria toxin is precipitated by lowering the pH of the solution. The isoelectric point of the toxin is near pH 4.1 (Pappenheimer 1937). It is likely that the precipitation of diphtheria toxin by acid is in part caused by denaturation of the protein which makes it less soluble (Eaton 1937). Several samples of tetanus toxin, purified by precipitation with cadmium chloride and fractionation with ammonium sulphate, were brought to pH 4.2 to 4.4 by the addition of dilute hydrochloric acid. A slight precipitate formed and was centrifuged down and redissolved in phosphate buffer at pH 7.0. The supernatant, adjusted to neutrality, and the redissolved precipitate were then tested for toxicity. It was found in all samples tested that at least 80 per cent of the toxin remained in the supernatant. The toxin found in the precipitate was probably carried down by adsorption to inactive protein.

Attempts to precipitate the toxin at a lower pH were unsuccessful. No further precipitate was formed when the solution of purified toxin was gradually acidified down to a pH of 2.4. At this pH the toxin is quite rapidly destroyed. Addition of 5 per cent trichloracetic acid (pH about 1.2) causes precipitation of some material which remains in solution at pH of 4.2, but the toxin is, of course, destroyed by the strong acid. These results indicate that tetanus toxin, when considerably purified, is not precipitated by acid at any pH at which the toxin is stable. Sommer (1937), Boivin and Izard (1937), and Ramon, Boivin, and Richou (1937) have purified tetanus toxin and anatoxin by precipitation with acid. It is probable that in these experiments the toxin was adsorbed to inactive protein precipitated by the acid.

PRECIPITATION OF PURIFIED TOXINS BY ALUM

Purified diphtheria toxin is readily precipitated by ammonium aluminium sulphate or ferric ammonium sulphate between pH 6.0 and 7.0. Tetanus toxin is precipitated almost completely from crude filtrates by ferric ammonium sulphate and incompletely by ammonium aluminium sulphate. After purification by the procedure described in this paper tetanus toxin is not readily precipitated by either reagent. The toxin was completely precipitated by ferric ammonium sulphate from one preparation, but in other experiments more than half of the toxin remained in the supernatant. By adding enough phosphate to cause the formation of a voluminous precipitate, more toxin could be brought down. These results suggest that tetanus toxin is precipitated only by adsorption to precipitates containing protein and insoluble phosphates.

DISCUSSION

Preparations of diphtheria and tetanus toxins purified to about the same degree by similar chemical procedures differ in the relative proportions of protein and non-protein nitrogen they contain, as well as in other chemical properties. Cadmium chloride under certain conditions precipitates proteoses as well as proteins. This accounts for the fact that one preparation is composed predominantly of protein and the other mostly of proteose or polypeptide.

The optimum conditions for the purification of the toxins were worked out empirically by trying various combinations of the reagents at different concentrations and values of pH and not by any preconceived idea as to the nature of the active fraction to be precipitated. As a result, the conditions for precipitation have been adjusted so that the most active diphtheria toxin is precipitated as a protein by cadmium chloride at a pH of 6.0 while tetanus toxin of maximum activity per mg. of nitrogen is precipitated in a slightly more alkaline pH range where proteoses are precipitated. The degree of purification attained indicates that the precipitation of both toxins is quite selective. It is likely that similar combinations of sodium citrate, sodium chloride, and cadmium chloride or other metallic salt could be used to selectively precipitate other proteins or polypeptides from broth filtrates, but the best combinations would not be the same in every case.

The adsorption of toxins, viruses, and other highly active agents to inactive precipitates is a well known phenomenon. This does not mean that all precipitates containing these agents are adsorption complexes. It is often possible to distinguish between adsorption and true precipitation of a chemical compound. Diphtheria toxin may be precipitated under relatively constant conditions by a variety of protein precipitants such as ammonium sulphate, phosphotungstic, or nucleic acids, lead acetate, uranyl acetate, potassium aluminum sulphate, acetone, or alcohol (Eaton 1936 a). The fact that highly purified diphtheria toxin behaves in the same way toward these reagents as toxin in the crude state indicates that the toxin itself is precipitated and not merely adsorbed to precipitates of inactive protein. Highly purified tetanus toxin differs from crude toxin in its behavior toward acid, alum, and possibly several other protein reagents. This indicates that adsorption to protein precipitates plays a part in the precipitation of tetanus toxin by protein reagents. The purified toxin is no longer precipitable by reagents which partially or completely precipitate it from crude filtrates. Diphtheria and tetanus toxins are salted out at approximately the same concentration of ammonium sulphate either in the crude or purified state.

Generalizations about the nature of bacterial toxins from one or two examples are not justified. Although the characterization of diphtheria toxin as a protein has apparently been well established, tetanus toxin may not be a true protein, and the scarlatinal toxin which is resistant to tryptic digestion may prove to be a nitrogenous carbohydrate (Hooker and Follensby 1934; Stock 1937). The diversity in the nature of toxins is, of course, limited by the number of substances that will act as antigens. At the present time the only pure substances which have been clearly shown to be antigens are proteins and certain complex carbohydrates. The possibility that tetanus toxin is a carbohydrate is apparently excluded by the fact that this toxin is destroyed by tryptic digestion.

SUMMARY

A simplified method for the purification of diphtheria and tetanus toxins by precipitation with cadmium chloride is described.

The preparations of the two toxins purified to about the same degree (separation of over 99 per cent of nitrogenous impurities) differ in the proportions of protein and non-protein nitrogen present. Although diphtheria toxin is precipitated by a variety of protein reagents, purified tetanus toxin is not precipitable by acid or alum.

The findings indicate that tetanus toxin differs in its chemical properties from diphtheria toxin which is a typical protein.

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OXIDATIONS PRODUCED BY HEMOLYTIC STREPTOCOCCI

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Received for publication April 23, 1938

Very little is known about the oxidations produced by hemolytic streptococci. Fujita and Kodama (1935) reported on the oxidation of glucose by *Streptococcus hemolyticus* and (because it lacked cytochrome and was cyanide insensitive) concluded that the respiration was catalyzed by Warburg and Christian's (1932) yellow ferment (phosphoriboflavin protein). Farrell's paper (1935) need not be discussed. He simply observed the reduction of a dye (indigotetrasulfonate) in the absence of oxygen, and from those observations drew conclusions on "dehydrogenations." The orientation of the reaction of an oxidizable substance may differ in the presence and in the absence of oxygen (for example, pyruvic acid, which is oxidized in the presence of oxygen, may split by dismutation in the absence of oxygen); reversible dyes may inhibit a dismutation in the absence of oxygen (Krebs (1937)); and, finally, these dyes may act as mediators of coupled oxidation-reductions, the dye being alternately reduced and oxidized. In such cases the observer would conclude by stating that no "dehydrogenation" took place. The species of bacteria grouped under the name hemolytic streptococci are of extreme interest. So far the group has defied classification by fermentation tests (Burger, 1907; Kendall, Day, Walker and Ryan, 1919; McClachlan, 1927). The rate of glucose breakdown by different strains of streptococci was also found to vary greatly when the bacteria were grown in broth containing glucose and phosphate (Hewitt, 1932).

A study of the oxidations produced by seven strains of hemolytic streptococci reported in this paper shows that different strains of hemolytic streptococci exhibit different metabolism; furthermore, that one strain may, on repeated transplantation in a blood-agar medium, change its oxidative enzymes.

EXPERIMENTAL

Seven strains of hemolytic streptococci were used in these experiments. They will, later on, be referred to only by the numbers given here:

No. 1. Erysipelas strain, Mosewitch; isolated in 1935, and since then subdivided into *1a*, the bacteria as studied during the first ten weeks of weekly transplant, and *1b*, the same bacteria later on.

No. 2. Scarlet fever strain, No. 2; isolated in 1924.
No. 3. Erysipelas strain, Valentine; isolated in 1934.
No. 4. Scarlet fever strain, Tyler; isolated in 1924.
No. 5. Scarlet fever strain, Greenwood; isolated in 1936.
No. 6. Scarlet fever strain, Wadsworth; isolated in 1924.
No. 7. Septicemia, Terihaj; isolated in 1937.

Most of these bacteria were isolated by Dr. George F. Dick.

After isolation, these streptococci were preserved in blood agar in sealed tubes, at room temperature. For the experiments reported here, they were grown in a beef-infusion blood-agar medium without glucose, in flat medicine bottles. After 24 hours' incubation at 37°, the bacteria were suspended in 0.9 per cent sterile NaCl, centrifuged, and suspended again in 0.9 per cent NaCl. The oxygen consumption and CO_2 production were measured at 38° by the usual Warburg-Barcroft manometric method. Two cubic centimeters of the bacterial suspension plus 0.5 cc. of 0.2 M phosphate buffer, pH 7.0, were used for each vessel. All these strains of hemolytic streptococci consumed negligible quantities of oxygen in the absence of oxidizable substances; thus, in every case, the vessel used as thermobarometer contained the bacterial suspension plus the buffer. The oxidizable substrates were kept in the side-arm of the Warburg vessels and were poured into the main vessel containing the bacterial suspension once temperature equilibrium was reached.

A pH value of 7.0 was chosen because this was found to be the optimum, on studying the influence of hydrogen ion concentration on the rate of oxidation of glucose, lactic acid, pyruvic acid, and glycerol by all the strains of streptococci used in these experiments. Volatile acids were determined by Friedemann's method (1937) adapted for the determination of small volumes. All the experiments reported here were repeatedly verified during a period of one year. Every bottle containing the bacteria was carefully examined before the experiments, those bottles which showed accidental contamination being rejected. The Warburg vessels, as well as all the pipettes used, were sterilized by keeping them overnight at 110°. The experiments never lasted more than two hours, at the end of which time smears were made from the bacterial suspensions in the Warburg vessels to verify lack of contamination.

THE OXIDATION OF CARBOHYDRATES

Extended experiments on the oxidation of carbohydrates were conducted, mainly with Streptococcus No. 1a. The hexoses were rapidly oxidized, the rate of oxidation decreasing in the following order: glucose, levulose, mannose, galactose. Arabinose was oxidized very slowly. Lactose was oxidized at a rate one-third that of glucose. Glyceric aldehyde was oxidized at about the same rate as lactose. Hexose monophosphate was oxidized slowly.[1] In table 1 are given the relative rates of oxidation of these carbohydrates, calculated by taking the rate of oxidation of glucose as 100. In all these experiments, the amount of oxidizable substrate was 0.03 m Mole, and the oxygen uptake was measured for one hour.

Oxidation of glucose by the different strains of hemolytic streptococci

In the absence of oxygen, glucose is fermented by streptococci mainly to lactic acid (Hewitt 1932; Friedemann).[2] In the presence of oxygen, washed streptococci oxidized glucose partially.

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[1] The sample of hexose monophosphate used in these experiments was kindly sent to one of us by Dr. C. V. Smythe.

[2] Personal communication by Dr. T. H. Friedemann.

TABLE 1

Oxidation of carbohydrates by Streptococcus hemolyticus no. 1a

As a rule, 1 mgm. of dry bacteria consumed 126 c.mm. O_2 per hour with glucose as substrate. Temp., 38°; pH, 7.01. Relative rate calculated by taking the rate of glucose oxidation as 100.

CARBOHYDRATE	RELATIVE RATE OF OXIDATION
Glucose	100
Levulose	92
Mannose	83
Galactose	77
Glyceric aldehyde	31
Lactose	30
Hexose monophosphate	13
Arabinose	1.3

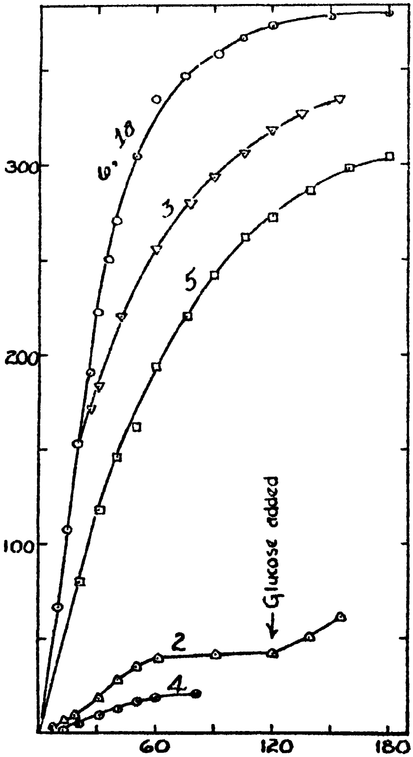

FIG. 1. OXIDATION OF GLUCOSE BY HEMOLYTIC STREPTOCOCCI

Amount of glucose, 0.01 mMole, Temp. 38°; pH, 7.01. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm. The numbers indicate the streptococcus species as numbered in the text.

Streptococci Nos. 1a, 3, 5, and 6 showed a rapid oxygen consumption up to 1.5 moles of oxygen per mole of glucose, the rate of oxidation becoming then gradually slower. Streptococci Nos. 2 and 4 oxidized glucose quite slowly, so slowly that the experiments had to be stopped before there was consumption of even 0.5 mole of oxygen per mole of glucose. The end of glucose oxidation can not be due to the accumulation of inhibitory oxidation products (as with pneumococci it is due to H_2O_2 accumulation), for the addition of more glucose started the oxygen uptake at the same rate as initially (fig. 1).

A glance at figure 1 shows that the strains here studied may be divided into two groups: those which oxidize glucose rather rapidly up to 1.5 moles of oxygen per mole of glucose; and those which oxidize glucose very slowly, the oxidation stopping with about $\frac{1}{2}$ mole of oxygen per mole of glucose. It must be emphasized that this difference in rates of oxidation is not due to the influence of environmental conditions, but rather to the characteristics of the bacteria, because the optimum pH value for the oxidation of glucose was found to be between 6.8 and 7.1 (the experiments were performed at a pH value of 7.0).

Effect of NaF and CH_2ICOOH on the oxidation of glucose

Glucose utilization by living cells seems to proceed according to varied mechanisms. Thus, the series of phosphorylations which occur in muscle during glycolysis do not occur in embryonic tissues, according to Needham and coworkers (1937). Furthermore, glucose may be oxidized either after its transformation to the C_3 compounds or after its transformation to hexose phosphates; it may even be oxidized directly. NaF (0.02 M) and iodoacetic acid (0.01 M), the well known inhibitors of glucose fermentation, inhibited the oxidation of glucose, the first completely, the second to the extent of 95 per cent (table 2). These inhibitions may be taken as indication that in the case of hemolytic streptococci glucose is first transformed into a triose or a hexose phosphate before it is oxidized. (It has been shown by Embden and Deuticke (1934) that the NaF inhibits the splitting of phosphoglyceric acid and that CH_2ICOOH inhibits the conversion of hexosediphosphate into the triose phosphates.) This inhibiting effect was observed in all the strains here reported.

Effect of HCN on the oxidation of glucose

Burnet (1927) was the first to report that *Streptococcus hemolyticus* could be grown in the presence of cyanide; he classified the bacteria as cyanide insensitive. His observations were confirmed by Fujita and Kodama (1935). The effect of HCN on the oxidation of glucose by hemolytic streptococci was found to vary according to the strain. In strains No. 1a, 5, and 6 the oxidation of glucose was not inhibited by HCN. It was partially inhibited in strains No. 1b, 4, and 2. In all these experiments the usual care was taken in order to avoid distillation of HCN over the KOH which was used to absorb CO_2 production.

TABLE 2

Effect of inhibitors on the oxidation of glucose by hemolytic streptococcus

INHIBITOR	O_2 UPTAKE IN 30 MINUTES		PER CENT INHIBITION
	Control	With inhibitor	
	c.mm.	*c.mm.*	
NaF (0.02 M) Strain No. 1	130.0	0	Complete inhibition
CH_2ICOOH (0.01 M) Strain No. 1	126.0	6.5	95
HCN (0.01 M) Strain No. 1a (April 23, 1937)	127.3	124.5	No inhibition
HCN, Strain No. 4	148.5	140.0	5.7
HCN, Strain No. 1b (November 11, 1937)	98.0	50.8	48.0
HCN, Strain No. 2	63.0	50.0	20.6
HCN, Strain No. 5	253.4	250.5	No inhibition
HCN, Strain No. 6	198.0	195.5	No inhibition

OXIDATION OF LACTIC ACID

The different oxidative behavior of various hemolytic streptococci can be better demonstrated by studying the oxidation of lactic and pyruvic acids and glycerol, for here there is a difference not only in rates of oxidation but, what is more significant, in the number of oxidations performed. Thus, some strains of hemolytic streptococci (Nos. 1a, 3, 5) oxidized lactic acid; others (2, 7, and 1b) did not oxidize lactic acid at all. Strain No. 4 oxidized it at a very slow rate (table 3). Those strains of hemolytic streptococci which did oxidize lactic acid at a measureable rate (Nos. 1a, 3, 5) used up one-half mole of oxygen per mole of lactic acid (fig. 2). These experiments could be interpreted as an oxidation of lactate to pyruvate:

TABLE 3

Oxidation of lactic acid, pyruvic acid, and glycerol by hemolytic streptococci

Amount of substrates, 0.02 mM; pH, 7.0; Temp., 38°

STREPTOCOCCUS NUMBER	OXYGEN UPTAKE IN 30 MINUTES		
	Lactate	Pyruvate	Glycerol
	c.mm.	*c.mm.*	*c.mm.*
1a	208	98.4	206
2	0	5.6	129
3	34	10.5	0
4	2	4.7	1,490
5	52	0	0
6	44	7.3	15
7	0		195

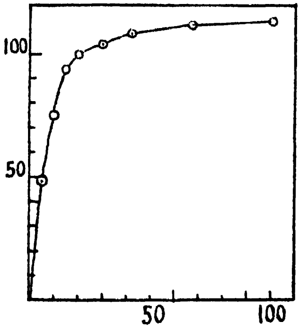

FIG. 2. OXIDATION OF LACTATE BY HEMOLYTIC STREPTOCOCCUS NO. 1a

Temp., 38°; pH, 7.01. Amount of lithium lactate, 0.01 mMole. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm. All strains of streptococci which oxidized lactate behaved similarly.

$$CH_3CHOHCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COCOOH + H_2O$$

A determination of the $\frac{CO_2\text{ output}}{O_2\text{ uptake}}$ quotient, however, showed that the lactate oxidation was accompanied by O_2 uptake and CO_2 output. 0.01 millimol of lactate used up on its oxidation 131 c.mm. of oxygen and produced 127.1 c.mm. CO_2. A respiratory quotient of one is obtained when lactic acid is oxidized to acetic acid and CO_2:

$$CH_3CHOHCOOH + O_2 \rightarrow CH_3COOH + CO_2 + H_2O$$

After steam distillation, the volatile acid distilled was identified as acetic acid by Krüger and Tschirch's (1929) color reaction with lanthanum nitrate and iodine. No formic acid or pyruvic acid was found. It may therefore be concluded that lactic acid is oxidized directly to acetic acid and CO_2. The factors responsible for the incomplete oxidation of lactic acid were not investigated.

Effect of inhibitors and dyes on the oxidation of lactic acid

Barron and Hastings (1933), on studying the oxidation of lactic acid by gonococci, found this oxidation performed through the coöperation of two factors: an oxidizing catalyst, probably a hemochromogen, and an activating enzyme. The first could be inhibited by HCN or H_2S and then replaced with a reversible dye of suitable oxidation-reduction potential. The second could be inhibited by a number of narcotics. The oxidation of lactic acid by streptococci was little affected by any of these inhibiting agents. Furthermore, the reversible dyes which increased lactic acid oxidation by gonococci had no effect or even inhibited its oxidation by hemolytic streptococci. Neither hydroxymalonic acid nor sodium arsenite inhibited the oxidation (table 4). In the last column of this table is given the inhibition produced by these substances on the oxidation of lactate by gonococci as found by Barron and Hastings (1933). It must be concluded from these experiments that the lactic-acid oxidizing enzyme of hemolytic streptococci is different from that usually found in other bacteria (gonococci, staphylococci, *Escherichia coli*).

OXIDATION OF PYRUVIC ACID

Streptococci No. 1 (both 1a and 1b) were the only strains which oxidized pyruvic acid rapidly. The other species of streptococci either oxidized it too slowly (Nos. 2, 3, 4, and 6) or did not oxidize it at all (No. 5) (table 3).

TABLE 4

Effect of inhibitors on the oxidation of lactate by Streptococcus hemolyticus

INHIBITOR	O_2 UPTAKE IN 30 MINUTES		INHIBITION	INHIBITION ON LACTATE OXIDATION BY GONOCOCCI
	Control	With inhibitor		
	c.mm.	*c.mm.*	*per cent*	*per cent*
HCN, 0.01 M	199.0	187.6	6.7	Complete
Phenyl urea, sat	190.0	117.0	38.4	52.0
Valeronitrile, 0.05 M	190.0	100.0	27.9	73.3
Hydroxymalonate, 0.05 M	190.0	137.0	8.7	
CH_2ICOOH, 0.01 M	68.8	62.9	8.7	62.0
Pyocyanine, 0.00015 M	133.1	118 0	11.3	Increase
Cresyl blue, 0.00015 M	150.0	79.0	47.3	Increase
Indigo tetrasulfonate, 0.00015 M	150.0	100.5	33.0	Increase
Sodium arsenite, 0.01 M	175.2	180 0	0	Increase
Octyl alcohol, sat	223.2	146.4	34.4	Complete

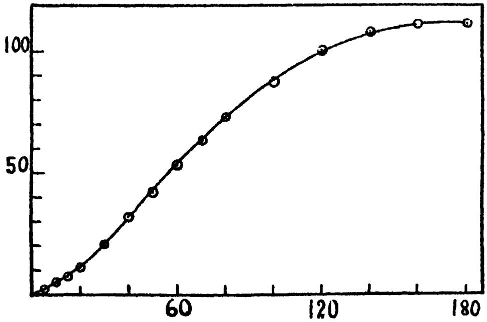

FIG. 3. OXIDATION OF PYRUVATE BY HEMOLYTIC STREPTOCOCCI, STRAIN 1a Temp., 38°; pH, 7.01. Amount of lithium pyruvate, 0.01 mMole. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm.

The oxidation of pyruvic acid by hemolytic streptococci, like that produced by gonococci (Barron (1936)), ended with an uptake of ½ mole of oxygen per mole of pyruvate (fig. 3). Since there was an output of 1 mole of CO_2 per mole of pyruvate, the oxidation can be expressed as follows:

$$CH_3COCOOH + \frac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$$

The validity of this equation was further demonstrated by titration of the acetic acid produced and its identification. One mole of acetic acid was formed per mole of pyruvic acid used by the bacteria. The metabolism of pyruvic acid by hemolytic streptococci follows a different path in the absence of oxygen. In such a case, as will be shown elsewhere, pyruvic acid splits into acetic acid and formic acid

$$CH_3COCOOH + H_2O \rightarrow CH_3COOH + HCOOH$$

Acetoacetic acid was not oxidized by hemolytic streptococci.

TABLE 5

Effect of inhibitors on the oxidation of pyruvate by Streptococcus hemolyticus

INHIBITOR	O_2 UPTAKE IN 30 MINUTES		INHIBITION	INHIBITION ON LACTATE O IDATION B GONOCOCC
	Control	With inhibitor		
	c.mm.	*c.mm.*	*per cent*	*per cent*
HCN, 0.01 M	135.8	71.7	70	95
NaF, 0.01 M	135.8	57.2	70	75
2,6-Dichlorophenol indophenol, 0.0001 M.	128.5	80.7	40.6	92

Effect of inhibitors

The oxidation of pyruvic acid by gonococci was inhibited by HCN, NaF, some reversible dyes, and a number of substances known as inhibitors of chain reactions (Barron (1936)). The oxidation of pyruvate by Streptococcus No. 1 was also inhibited by these substances, although to a less degree (table 5).

OXIDATION OF GLYCEROL

As in the case of the other two metabolites, some strains of hemolytic streptococci oxidized glycerol quite vigorously (Nos. 1b, 2, 4, and 7); Strain No. 6 oxidized it very slowly; Strains Nos. 3 and 5 failed to oxidize glycerol at all (table 3). Those strains which did oxidize glycerol, oxidized also ethyl, propyl, and butyl alcohol; methyl alcohol, glycol, mannitol, erythritol, and sorbitol were not oxidized.

The glycerol-oxidizing enzyme in these bacteria was found to be the most stable of all oxidizing enzymes. Suspensions of washed bacteria in 0.9 per cent NaCl plus a few drops of toluol could be kept at 3° for as long as 7 days without destruction of the glycerol-oxidizing enzyme. These suspensions of toluol-treated bacteria oxidized glycerol rapidly with an oxygen uptake of 1 mole per mole of glycerol (fig. 4) and a CO_2 output of 0.5 mole. No acid was formed by the oxidation of glycerol; H_2O_2 formation was detected by its color reaction with titanous sulfate. There was no acetaldehyde formation. On titration with Campbell's method (1926) for the determination of dihydroxyacetone (which is not specific because glyceraldehyde can also be titrated

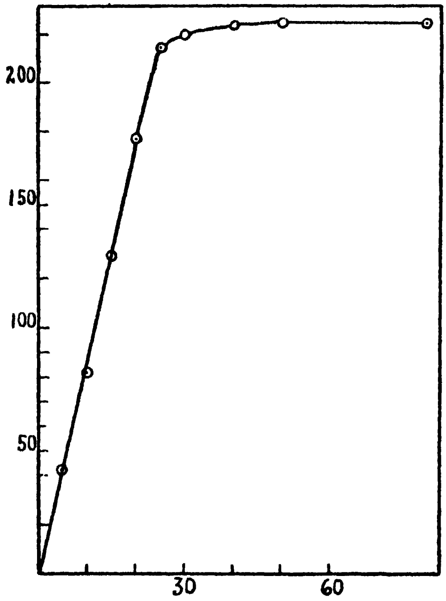

FIG. 4. OXIDATION OF GLYCEROL BY HEMOLYTIC STREPTOCOCCI, STRAIN 1a
Temp., 38°; pH, 7.01. Amount of glycerol, 0.01 mMole. Washed bacteria, kept at 3° for 30 hours. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm. The total CO_2 output was 110 c.mm. *RQ* = 0.5. All the strains which oxidized glycerol behaved similarly.

with phosphomolybdic acid reagent) there was found 0.5 mole per mole of glycerol oxidized. It is therefore likely that glycerol is oxidized by streptococci according to the following equation:

$$2\,CH_2OHCHOHCH_2OH + 2\,O_2 \rightarrow CH_2OHCOCH_2OH + CH_2OHCH_2OH + CO_2 + H_2O_2 + H_2O$$

An alcohol was found in the oxidation product, but whether it is glycol or not could not be established, since there are no methods to titrate glycol in the presence of other alcohols.

The oxidation of glycerol was not inhibited by HCN (0.01 M); it was inhibited almost completely by NaF (0.01 M), CH_2ICOOH (0.01 M) and pyrophosphate (0.025 M).

Formic, acetic propionic, butyric, succinic, oxalic, glycolic, citric, glyceric, α- and β-glycerophosphoric acids were not oxidized at a measurable rate by hemolytic streptococci. The following amino acids were not oxidized: alanine, asparagine, Na glutaminate.

VARIATIONS IN THE RATE OF OXIDATION OF GLUCOSE, GLYCEROL, AND LACTATE BY STREPTOCOCCUS HEMOLYTICUS NO. 1 AT DIFFERENT PERIODS

Burger (1907) pointed out that the fermentative power of a strain of streptococcus could be changed by repeated transplantation into a medium containing different sugars. This ability of bacteria to modify the nature of their enzymes when grown in the presence of special substrates has indeed been utilized by Dubos and Avery (1931) to develop bacteria able to split the capsular polysaccharides of Type III pneumococcus, and by Miller and Dubos (1936) to adapt certain bacteria to the decomposition of creatinine. Streptococcus No. 1 changed its oxidative properties while being grown in the same nutrient media, as shown in a study of the rates of oxidation of glucose, glycerol, and lactic acid at different periods from June 17, 1937, to February 6, 1938. The cultures were grown weekly in the usual medium, blood agar, and the experiments were performed as previously with 24 hour cultures. At first the rates of oxidation decreased in the following order: lactate > glucose > glycerol. Gradually the bacteria lost the power of oxidizing lactic acid while their power to oxidize glycerol increased. On August 26, i.e., after ten transplantations in the blood-agar medium, these streptococci lost entirely the power to oxidize lactic acid, while retaining the power to oxidize glucose and glycerol. From that date on, the oxidizing enzymes of these bacteria remained unchanged. In figure 5 the relative oxidizing power of Streptococcus No. 1 towards glucose, glycerol, and lactate has been plotted, the rate of glucose oxidation having been taken as 100.

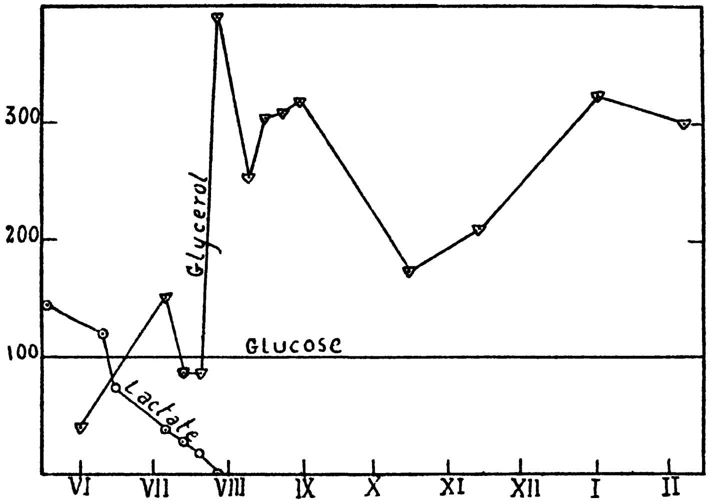

FIG. 5. RELATIVE RATES OF OXIDATION OF GLUCOSE, GLYCEROL, AND LACTATE BY STREPTOCOCCUS HEMOLYTICUS NO. 1, AS DETERMINED DURING A PERIOD OF SEVEN MONTHS

The rate of glucose oxidation has been taken as 100. Abscissa, time in months. Ordinate, relative rates of oxidation.

This change in oxidation enzymes was not confined to the loss of the lactic acid oxidizing enzyme, but extended to the mechanism of glucose oxidation. Thus the bacteria during the first weeks (Streptococcus No. 1a) appeared to oxidize glucose via lactic acid with acetic acid and CO_2 as end products, according to the equation:

$$C_6H_{12}O_6 \xrightarrow{\text{fermentation}} 2\,C_3H_6O_3;$$

$$2\,C_3H_6O_3 + 2\,O_2 = 2\,C_2H_4O_2 + 2\,CO_2 + 2\,H_2O$$

The oxidation of glucose used up 2 moles of oxygen per mole of glucose, with a CO_2 production of 2 moles; furthermore, the rate of lactate oxidation was greater than that of glucose. When the streptococci lost the power of oxidizing lactate, the oxidation of glucose ended with an oxygen uptake of ½ mole per mole of glucose. The renewal of oxygen uptake upon the addition of more glucose shows that this process was stopped by lack of oxidizable substance and not by death of the bacteria (fig. 6).

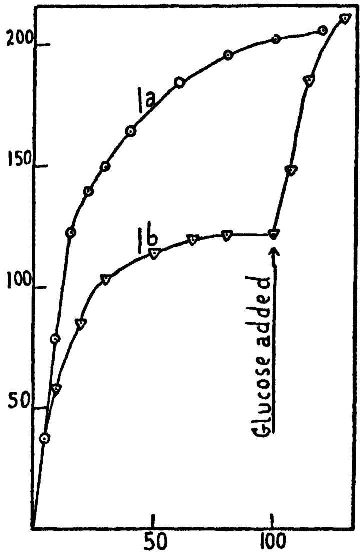

FIG. 6. THE OXIDATION OF GLUCOSE BY STREPTOCOCCUS HEMOLYTICUS NO. 1
Ia. During the first ten weeks of weekly transplantation in blood-agar. *Ib.* After seven months of frequent transplantations. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm.

The first oxidation was insensitive to HCN; the second was partially inhibited by HCN (0.01 M).

It must be emphasized that the loss of the ability to oxidize lactic acid, the fundamental change, did not occur *suddenly* but *gradually*, and that the streptococci retained their pathogenic power as shown in tests with mice. An objection to the theory of strain change might be the assumption that the original culture contained two strains of streptococci, one similar to strains 1a, 3, 4, 5, and 6, able to oxidize lactic acid; and the other, similar to strains 2 and 7, unable to oxidize it, and that in the process of repeated transplantations the latter strain became more and more dominant. This hypothesis would not explain the fact that the rate of pyruvic acid oxidation *remained unchanged* throughout the whole time the experiments were performed, namely, one year.

DISCUSSION

Oxidations produced by hemolytic streptococci, although small in number, vary greatly from one strain to another. These differences are particularly striking in comparing the oxidations produced by hemolytic streptococci isolated from cases of scarlet fever. Streptococcus No. 2 oxidized glycerol almost exclusively, for the rate of glucose and pyruvic acid oxidation was small, and lactic acid was not oxidized. Streptococcus No. 6 oxidized glucose at high speed, and lactate at lower speed; glycerol and pyruvate were oxidized very slowly. Streptococcus No. 5 oxidized only glucose and lactate; glycerol and pyruvate were not oxidized (table 6). The relative rates were calculated by making an average of all the experiments performed with these bacteria during one year. If such varied forms of oxidation are found in hemolytic streptococci isolated from scarlet fever,

TABLE 6

Relative rates of oxidation produced by seven strains of hemolytic Streptococcus

For the calculation of these rates, the rate of glucose oxidation was taken as 100

STREPTOCOCCUS NUMBER	RELATIVE RATES OF OXIDATION			
	Glucose	Glycerol	Lactate	Pyruvate
1a (erysipelas)		40	144	35
1b (erysipelas)		174	0	36
2 (scarlet fever)	Rate too slow	642	0	26
3 (erysipelas)		0	29	3
4 (scarlet fever)	Rate too slow	1,490	25	0
5 (scarlet fever)		0	45	0
6 (scarlet fever)		7	20	1
7 (septicemia)		327	0	

it is easy to understand the failure of the numerous attempts to classify the whole group of bacteria collected under the name of hemolytic streptococci. This failure is more apparent in Streptococcus No. 1, an organism isolated by Dr. Dick in 1935 from a case of erysipelas and kept for two years in his laboratory. It changed its metabolism on repeated transplantation. An organism which oxidized lactate changed into one which did not oxidize it.

It is quite possible that these differences of metabolism may also be found in other bacteria. For example, Sevag and Neuenschwander-Lemmer (1936) reported that *Staphylococcus aureus* (grown in bouillon) did *not* oxidize pyruvic acid. Krebs (1937) found that *Staphylococcus aureus* grown in broth agar oxidized pyruvic acid to about half of complete oxidation. Barron and Lyman (to be published) on studying five different strains of *Staphylococcus aureus* found that one of them oxidized pyruvic acid to completion (CO_2 and H_2O) while the other four oxidized it readily to $\frac{3}{5}$ of completion.

It must be emphasized that, though the oxidation of lactic acid by α-hydroxyoxidase from gonococci and hemolytic streptococci ends with an oxygen uptake of $\frac{1}{2}$ mole per mole of lactic acid, the two oxidation processes are different. The first oxidation, a reversible process, ends with the formation of pyruvic acid; the second process is a direct oxidation to acetic acid and CO_2, a decarboxylative oxidation.

SUMMARY

The metabolism of hemolytic streptococci, as studied in seven different strains for a period of one year, showed a great variation in the number of oxidations produced by the bacteria, as well as in the relative rates of oxidation. One group oxidized glucose, lactic acid, pyruvic acid, and glycerol; a second group oxidized glucose, lactic acid, and glycerol; a third group oxidized only glucose and lactic acid. Furthermore one strain, on continued transplantation, lost *gradually* the power to oxidize lactic acid while retaining its pathogenicity and the power to oxidize pyruvic acid.

We express our thanks to Dr. George F. Dick for kindly giving us his collection of hemolytic streptococci for use in these experiments.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARYLAND BRANCH

Panel Discussion on Coliform Bacteria, May 10, 1938

Contributors: Harry E. Jordan, Carl J. Lauter, and C. A. Perry

Escherichia coli Versus the Coliform Group of Bacteria as an Index of Fecal Pollution in Shellfish and the Value of the Eijkman Test as a Primary Test for Escherichia coli. *C. A. Perry*, Maryland State Department of Health.

It was pointed out that while the coliform group of bacteria offers a satisfactory test to determine the freedom of drinking water from ordinary pathogenic bacteria of the enteric group, the use of one of the simpler tests (such as brilliant-green lactose bile medium) would obviate the need for confirmation, would be sufficiently accurate and sensitive for general purposes, and would make results available earlier, while the time saved could be profitably used, when necessary, for the determination of *Escherichia coli.* Information on *Escherichia coli* pollution would be of considerable value in many small drinking water supplies which it is not practical to treat or where treatment is not warranted on the basis of sanitary inspection.

However valid the coliform group may be for drinking waters which may be filtered, chlorinated or protected, the use of the group has not been found satisfactory for estimating pollution in shellfish in which certain coliform bacteria especially of the *Aerobacter cloacae* type have a natural habitat and grow to large numbers under suitable temperatures and in the absence of pollution.

Escherichia coli has been found to correlate closely with pollution as estimated from sanitary survey data and to be a satisfactory index for estimating probable fecal pollution in shellfish.

Primary culture in a suitable medium at 45.5°C. has been found a practical test for *Escherichia coli.* Most *Citrobacter* and *Aerobacter* strains are inhibited under these conditions with the result that a great deal more *Escherichia coli* may be isolated than is possible with lactose broth at 37°C. This is particularly true where relatively large numbers of *Citrobacter* or *Aerobacter* types are present. The original Eijkman medium of 1904 has been greatly modified and a simple, effective medium developed. Lactose has recently been found superior to glucose in the Modified Eijkman medium in the recovery of *Escherichia coli* from raw sewage. It is believed that by further study this test may be made even more practical.

SOUTHERN CALIFORNIA BRANCH

(In Cooperation with Pacific Division of the A. A. A. S.)

San Diego, California, June 24, 1938

The Rôle of Marine Microörganisms in the Formation of Primary Film on Submerged Surfaces. *W. Forest Whedon*, Scripps Institution of Oceanography, La Jolla, California.

The work to be reported here has

been carried on by the Bureau of Construction and Repair, a division of the United States Navy Department, with the coöperation of the Scripps Institution of Oceanography, La Jolla, California.

The marine microörganisms which contribute to the formation of primary film on submerged surfaces include a great many species of bacteria, and several species of protozoa, namely, suctoria, ciliates, and amebae. Since only a comparatively few free-living bacteria occur in the sea and practically all others are associated with organic detritus, an understanding of the extensiveness of this relationship in primary film formation assumes an aspect of prime importance. The evidence presented by this investigation has shown that the products which result from the breakdown of organic detritus tend to adhere to the surface of a submerged object and to spread out over its surface. The bacteria multiply quite rapidly, following attachment, and spread out over the surface with the flow of the dissolving organic matter, thus forming a film.

THE INFLUENCE OF TEMPERATURE ON THE ACTIVITIES OF MARINE BACTERIA. *Claude E. ZoBell and Catharine B. Feltham*, Scripps Institution of Oceanography, University of California, La Jolla, California.

Marine bacteria have been found to be hypersensitive to temperature changes. Even the short exposure to the pouring temperature of nutrient agar (40 to 45°C.) during plating procedures is lethal to many marine bacteria and some lose their viability when exposed to temperatures of no higher than 30°C. for 10 minutes. The cause of the extreme heat-sensitivity is not well understood, particularly in view of the fact that within a few generations most of the marine bacteria can be acclimatized to tolerate temperatures as high as 55°C. for ten minutes.

The optimum temperature for the multiplication of these bacteria is between 15° and 25°C. That for the activity of their enzymes is between 25° and 35°C. No psychrophiles have been observed, although most of the bacteria are habitats of a marine environment which is perpetually colder than 4°C. However, nearly all of them will multiply at near zero temperatures. Some form colonies on plates incubated at −4 to −7°C. The ability of marine bacteria to grow at low temperatures accounts for the fact that marine food products may slowly undergo decomposition even at refrigeration temperatures.

PRECURSORS TO THE FORMATION OF CREATININE BY BACTERIA. *T. D. Beckwith and C. H. Fish*, Department of Bacteriology, University of California at Los Angeles.

Creatinine can be produced from peptone by a variety of bacteria but the peptone must be considered as a complex structure involving many factors. Further consideration has proven that creatinine may be formed through bacterial action from various amino acids when glucose is present. In seeking for its precursors, creatinine has been treated as a compound containing two critical portions which are (1) acetic acid and (2) guanidine. It has been shown that glycine, urea and glucose when under the influence of *Bacillus proteus* give rise to considerable concentrations of creatinine. Other amino acids than glycine supply either the acetic acid or urea necessary for the reaction. Added evidence regarding the importance of urea is presented by treatment of arginine by an activator of arginase. For production of creatinine by *B. proteus*, urea may not be substituted by a salt of ammonium. The hexose glucose is the form of carbohydrate most readily available for use by the organism in order that creatinine may appear. The precursors of creatinine, as produced by *B. proteus*, are acetic acid and urea.

THE EFFECT OF INDOL 3 ACETIC ACID UPON MULTIPLICATION OF B. COLI-COMMUNIS AND B. TYPHOSUS. *T. D. Beckwith and E. H. Geary*, Department of Bacteriology, University of California at Los Angeles.

For these experiments the two organisms named were chosen since *Bacillus coli* produces both indole and acetic acid while *Bacillus typhosus* does not form indole from tryptophane. With the organism of typhoid, concentrations of the heteroauxin lying between 1:3,000,000 and 1:25,000 are stimulating to multiplication while with *Bacillus coli-communis* the zone of stimulation spreads from 1:3,000,000 to 1:5,000. Concentrations of 1:100 and greater were found to be completely toxic for both organisms and some growth inhibition was noted at the concentration of 1:1,000.

THE PROTECTIVE ACTION OF TYPHOID BACTERIOPHAGE ON EXPERIMENTAL TYPHOID INFECTION IN MICE. *Roy T. Fisk*, University of Southern California School of Medicine and Los Angeles General Hospital.

A GENERALIZED ASPERGILLUS INFECTION OF PENGUINS. *Leo F. Conti*, San Diego Zoological Research Laboratory, San Diego.

Advanced pulmonary Aspergillosis in Humboldt penguins (*Spheniscus humboldtii*) from Peru, found on autopsy twenty-nine days after removal from their natural habitat, may indicate the infection as originating in nature.

Deflation of all air sacs prior to death produces what erroneously appears to be a loss of weight.

The clotting time of blood in penguins has not been recorded, its rapid fibrination making the blood-counting procedure extremely difficult.

The intradermal tuberculin test reported by other workers as producing a positive reaction in pulmonary Aspergillosis of humans, was negative in penguins and an experimentally infected domestic chicken and rabbit.

Changes noted in the differential blood count of a domestic chicken artificially infected with Aspergillosis revealed an increase in the eosinophil and lymphocyte count. Leucocytosis as found in tuberculosis of chickens was observed only between the 15th and 60th days following inoculation.

Further blood studies in test animals experimentally infected with *Aspergillus fumigatus* seem warranted.

EXPERIMENTS ON ANTIRABIC IMMUNIZATION. *Anson Hoyt*, University of Southern California School of Medicine.

STUDIES ON THE NEUTRALIZING ANTIBODY IN THE SERUM OF POLIOMYELITIS PATIENTS AT ONSET AND RECOVERY. *John F. Kessel and Fred D. Stimpert*, University of Southern California School of Medicine and Los Angeles General Hospital.

COMPARATIVE METABOLISM OF THE R AND S VARIANTS OF LACTOBACILLUS PLANTARUM. *Ralph L. Tracy*, University of California School of Medicine.

FIELD CONTROL ROUTINE FOR OBTAINING SANITIZATION OF EATING AND

DRINKING UTENSILS. *R. V. Stone*, Los Angeles County Health Department.

In 1935, a sub-committee of the A. P. H. A. was appointed to study the problem of sterilization of eating utensils.

In March, 1937 the A. P. H. A. year book carried tentative standard procedures developed by the committee. Essentially these included a maximum allowable count of 50,000 colonies per ml. of dish water; a bacterial count not to exceed 500 colonies per rim of a drinking glass; a temperature of wash water from 110 to 120°F.; a rinse water of not less than 170°F. or a bath with a chlorine content of from 50 to 200 p.p.m. kept at a warm temperature.

The word "sterilize" implies an actual sterility attainment. This is seldom realized. The use of the word "sanitization" more nearly describes the objective in view.

Wash and rinse water bacterial counts occur, not infrequently, in the millions, both on the Atlantic and Pacific Coasts.

Temperatures of wash waters and rinse waters are usually too low (under 110°F.). Wash waters are surprisingly dirty—chlorine rinses too seldom used.

Data gathered by the Los Angeles County Health Department have led to the development of a practical control routine. This is not the final answer to the problem; however, it provides the best field procedure so far of real value to us. It includes three things:

a. A thermometer for checking temperatures of wash waters (a minimum of 120°F. should be demanded).

b. A sediment tester for the physical demonstration that dirty dish water *is* dirty water. (Clean water should be demanded.)

c. A chlorine tester for checking the strength of chlorine rinses (100 p.p.m. should be demanded as the minimum allowed). A chlorine rinse provides a "safety zone" which is needed when one observes the usual failure of hot water heaters to maintain efficiency during rush periods.

SENSITIZING BACTERIAL SPORES TO HEAT BY EXPOSING THEM TO ULTRAVIOLET LIGHT

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Received for publication May 4, 1938

Among the many physico-chemical changes produced in proteins by ultraviolet radiation, the changes that affect stability to heat are of particular interest to the biologist. With those rare exceptions noted by Bovie and Woolpert (1924) and Clark (1925) the heat-coagulation temperature of probably all proteins is lowered by the absorption of ultraviolet light. In a study of twelve purified proteins Stedman and Mendel (1926) found that ultraviolet light from a quartz-mercury lamp reduced the temperature of coagulation from 8.5° to 41°C.

Since the killing of living organisms by heat is accompanied by coagulation of the protoplasm it is reasonable to suppose that their heat-resistance would be decreased by ultraviolet radiation. Despite the interesting implications involved, only Bovie and Klein (1919) and Bovie and Daland (1923) have studied the heat-sensitization phenomenon with living microörganisms. Working with Paramecia they observed that sublethal exposures to ultraviolet light, transmitted by fluorite, so affected the organisms that they subsequently died at temperatures which were optimal for the untreated organisms. These striking effects were tacitly assumed to be caused by those rays of very short wave length (Schumann) which are transmitted only by fluorite. While this assumption was undoubtedly correct, these experiments might very naturally lead to the belief that the Schumann rays alone were possessed of heat-sensitizing power. This circumstance, together with certain technical difficulties involved in the generation and transmission of Schumann rays, is perhaps chiefly responsible for the absence of additional work on this subject.

Consideration of these facts and of the potentialities inherent in the heat-sensitizing principle led us to study the action of ultraviolet rays upon the heat-resistance of bacterial spores. The report which follows presents the results obtained in this investigation.

EXPERIMENTAL

Cultures and Methods

Three cultures (*Bacillus cohaerens*, *Bacillus albolactis* and CC) were used. The first two were obtained from the American Type Culture Collection. CC, an aerobic spore-former belonging to the *Bacillus mesentericus* group, was isolated in our laboratory from evaporated milk. The cultivation and preparation of the test suspensions have been detailed in a previous paper, by Curran and Evans (1937). The stock and test suspensions when not in use were held in the ice chest.

Two sources of ultraviolet light were used. The first was a cold-mercury-in-quartz lamp of the orificial type. Tests by the National Bureau of Standards upon the spectral energy distribution of this lamp have indicated that at least 95 per cent of the total radiation of all wave lengths less than and including the line at 3,130 Å. is contained in the emission line of mercury vapor at 2,537 Å. Four milliliter quantities of the spore suspension at 30°C. were irradiated in a small quartz balloon flask (18 ml. capacity), placed 2¾ inches from the burner. The flask was equipped with a quartz stirrer which provided rapid and uniform agitation. After the exposure to ultraviolet light, equal portions of the spore-suspension were mixed on plates with 15 ml. of agar of the desired composition. Colony-counts were made after 48 hours of incubation at the optimum temperature of the organism. A magnifying glass was used in counting and the figures represent the averages of triplicate plates. From time to time the plates were returned to the incubator, and reexamined 5 days later to check the possibility of slowly developing colonies. The intensity of the cold-quartz radiation was measured by exposing a sensitive thermopile at a fixed distance from the burner and noting the galvanometer deflections. These values were then converted into absolute energy units by reference to a standard of radiation.

The second source of ultraviolet light was a hydrogen-discharge tube (15 by 1⅛ inches) fitted with ring electrodes of aluminum. In one end of the tube was sealed a fluorite disc (2 mm. thick) and in the other end a quartz disc of the same thickness. The discs were equi-distant from the electrodes. The tube was excited by a 450-watt luminous tube transformer operating on a 110-volt alternating current of 60 cycles. The current passing through the discharge tube was 33 milliamperes and the drop of potential across the tube was 1,570 volts. The intensity of the radiations transmitted by the tube was not measured. Exposures to this light source were made as follows. A small platinum loopful of a heavy aqueous suspension of the spores was spread uniformly over a 15 x 5 mm. area of a sterile coverslip until dry. The dried film was then applied directly to the window of the discharge tube and exposed. The temperature of the discharge tube windows did not rise perceptibly during the exposures recorded. The spores were recovered in 5 ml. of sterile distilled water; the process of loosening the film from the glass was facilitated by the use of a small, sterile, rubber-tipped glass rod. The suspensions were then thoroughly agitated and plated. The count so obtained served thereafter as a guide for the dilution required to equalize the number of viable cells in the several samples.

Aliquot portions of the standardized samples were heated at 98°C. for 4 minutes and plated.

The medium used was standard nutrient agar of the following composition: 0.5 per cent peptone (Difco), 0.3 per cent beef extract (Liebig's), 0.5 per cent sodium chloride, 1.5 per cent agar (granulated). This basic medium was enriched with either blood or glucose, depending on the nature of the treatment and the enrichment requirements of the organism (See Curran and Evans, 1937).

When bacteria are exposed to both heat and ultraviolet radiation, of constant intensity for the same period of time, the order in which the two treatments are applied should not appreciably affect the number of survivors, if sensitization to neither heat nor light occurs, or if sensitization to both heat and light occurs to the same extent. However, consistent differences in the number of survivors under these conditions do occur and must be attributed to differences in the sensitizing power of the initial lethal agent. The results presented in Table I were obtained when spores uniformly dispersed in distilled water were exposed to radiation from a quartz-mercury lamp and heat of constant intensity in the order, and for the periods, indicated. Changes in the reaction of the spore suspension during irradiation did not exceed 0.1 pH; hence this factor may be disregarded in the interpretation of results. These data clearly show that the spore-

TABLE 1

The effect of heat (98°C.) and ultraviolet light (21 Ergs/mm^2/sec) on viability of bacterial spores, according to the sequence of treatment and time of exposure

KIND OF SPORES, AND TIME EXPOSED TO HEAT AND LIGHT	VIABLE SPORES SURVIVING PER MILLILITER WHEN THE SEQUENCE OF TREATMENT WAS:		SURVIVOR RATIO (H/L : L/H)
	Heat/Light	Light/Heat	
*B. cohaerens:**			
1½ minutes	900,000	843,000	1.0:1.0
3 minutes	205,000	48,000	4.2:1.0
5 minutes	3,460	420	8.2:1.0
7 minutes	32	13	2.4:1.0
CC:†			
2 minutes	400,000	346,000	1.0:1.0
3 minutes	308,000	138,000	2.2:1.0
6 minutes	23,400	4,900	4.7:1.0
9 minutes	392	270	1.0:1.0
B. albolactis:‡			
1 minute	65,000	64,000	1.0:1.0
2 minutes	2,920	1,470	1.9:1.0
3 minutes	556	130	4.2:1.0
3½ minutes	186	30	6.2:1.0

* Untreated control contained 1,900,000 viable spores per ml.

† Untreated control contained 2,100,000 viable spores per ml.

‡ Untreated control contained 350,000 viable spores per ml.

killing action of any given combination of heat and ultraviolet light is materially influenced by the order of their application.

When the periods of exposure were short and the mortality relatively low, the order of treatment had but little influence upon the number of spores which survived; a slight but distinctly greater mortality resulting when heat succeeded irradiation. With lengthening of the exposure periods and consequent re-reduction in number of survivors, the order of treatment became of increasing significance. Comparative differences are indicated by changes in the survivor ratio. Light preceding heat was always more destructive than heat preceding light. As may be noted in table 1, the survivor ratio increased with length of the exposures until maximum sensitization was attained. With some species, this was followed by a reduction in the survivor ratio; with one species such an effect was not observed.

The spores of the three species reacted in essentially the same way to the combined action of irradiation and heat. The sensitizing effect was greatest with the spores of *B. cohaerens* in which the survivor ratio at its maximum was more than 8:1. The increased mortality attributable to sensitization, manifestly represents only a very small part of the total population but it is significant that the spores most easily sensitized are those of high resistance.

The change produced in spores by exposure to ultraviolet light is apparently irreversible since their tolerance to heat was found to be independent of the time which elapsed between the two treatments.

The heat-sensitizing action of ultraviolet light is again brought out in table 2. Under the conditions of the experiment, irradiation for 2 minutes was about equal to heat for 5 minutes in sporocidal action. By making a suitable dilution of the untreated suspension, a control suspension of spores could be obtained in which the number of viable spores was comparable with the number surviving a 5 minute heating period or 2 minutes' irradiation. To the control suspension was added a sufficient amount of heat-killed spores to make the total number of living and dead spores essentially the same in the three samples.

Under these conditions, when an irradiated suspension was given a second exposure to light only 100,000 spores survived. When the suspension was first heated and then irradiated, over six times as many spores survived and this number was about equal to the number that survived a single exposure to ultraviolet light when the suspension was neither heated nor irradiated previously. The spores previously heated were, therefore, no more sensitive to irradiation than spores not previously heated or irradiated. When heated spores were given a second heating the mortality was somewhat greater than for the light-heat combination, but the control spores in this instance were considerably more resistant to heat than were the spores that had been irradiated.

TABLE 2

The effect of ultraviolet light (incident energy 22.2 Ergs/mm^2*/sec.) and of heat (98°C.) upon spores of B. cohaerens*

VIABLE SPORES PER MILLILITER IN UNTREATED SUSPENSION	VIABLE SPORES PER MILLILITER WHEN THE SEQUENCES AND TIMES OF TREATMENT WERE					
	First treatment	Time	Count	Second treatment	Time	Count
		min.			min.	
2,700,000	Light	2	770,000	Light	2	100,000
				Heat	5	230,000
	Heat	5	860,000	Light	2	650,000
				Heat	5	180,000
	Dilution		840,000	Light	2	540,000
				Heat	5	450,000

It is apparent that spores which survive lethal heat or lethal ultraviolet light are sensitized to that particular form of energy. Although the results are somewhat variable the general relationships were consistently obtained under conditions of adequate irradiation. Thus, where heat and ultraviolet light of about the same sporocidal power are concerned, sensitization to light is best produced by light, and sensitization to heat is best produced by heat. When combinations are involved, light sensitizes to heat less than heat does to heat. Since heated spores are no more sensitive to irradiation than spores that have not been first heated or irradiated, heat may be regarded as having little or no light-sensitizing action. In the foregoing experiments the spores were exposed in a fused quartz vessel, hence the effects observed were produced by rays of wave lengths longer than 2,000 Å.

Since the effects described by Bovie *et al.* were believed to be caused by ultraviolet rays of very short wave length it seemed of particular interest to compare the heat-sensitizing action of long and very short ultraviolet rays. In order to accomplish this, the spores were dried on sterile coverslips and during exposure pressed tightly against the fluorite and quartz windows of a hydrogen discharge tube. After exposure, the spores were collected and treated as previously described. Ozone effects may be considered negligible owing to the exclusion of air between the discharge tube windows and the spores.

Examination of table 3 shows that spores not previously exposed to ultraviolet light were clearly much more resistant to heat than those previously exposed to light passing through fluorite or quartz. The objection may be made that spores which survive a destructive influence might reasonably be expected to be so weakened that they would die more rapidly when exposed to another destructive influence. However, reasonable as this supposition may seem, it is not universally true. As pointed out in connection with the study of the light-heat sequence in table 2, spores which survived heat were no more sensitive to ultraviolet light than those not previously exposed to heat. We believe, therefore, that irradiated spores are more susceptible to heat because of a specific change in the condition of the cells produced by the ultraviolet rays. The heat-sensitizing effects of the rays passing through fluorite and quartz as shown in the table are not directly comparable because of differences in transparency of the two substances. With equal exposures somewhat more energy would be emitted at the fluorite window.

In an effort to obtain results which would serve as a more satisfactory basis for comparison, the exposures through quartz were lengthened by 5 second increments up to 30 seconds. Examination of the results obtained shows that the light transmitted by quartz was much less effective in sensitizing the spores to heat even with much longer exposures. These effects are best observed in the percentage reduction values. Only when the exposure through quartz was three times that through fluorite were the heat-sensitizing effects similar. From this it is evident that ultraviolet rays of very short wave length have much greater heat-sensitizing action upon spores than those transmitted by quartz. The sample of fluorite used was transparent to 1,250 Å.

TABLE 3

The influence of ultraviolet light transmitted by quartz and fluorite upon the heat-resistance of B. cohaerens spores

ULTRAVIOLET TREATMENT OF B. COHAERENS IN ORIGINAL SAMPLES	VIABLE SPORES PER MILLILITER		PERCENTAGE REDUCTION BY HEATING
	In original samples after irradiation	In irradiated samples after heating for 4 minutes at 98°C.	
			per cent
No treatment (control)	368	181	50.8
Fluorite, for 10 seconds	445	40	91.0
Quartz:			
For 10 seconds	430	153	64.4
For 15 seconds	389	94	75.8
For 20 seconds	421	77	81.7
For 30 seconds	407	40	90.1
Fluorite, plus quartz for 10 seconds	380	151	60.2
Quartz, plus quartz for 10 seconds	385	151	52.3
Fluorite, for 10 seconds at 5 mm	440	211	52.0
Quartz, for 10 seconds at 5 mm	430	201	53.2

Crystal quartz of the thickness used is transparent to 1,600 Å., according to Lyman (1914), hence the effects observed are to be attributed to the Schumann rays through a spectral range of 350 Å.

In this study some exposures were made in which a second quartz disc 2 mm. thick was interposed between the spores and the discharge tube windows. So treated, the heat-sensitizing effects in the two combinations were almost identical and both somewhat less than those obtained with light transmitted by the single quartz disc. The second quartz disc absorbs completely the shorter rays passing through fluorite and the exclusion of these rays reduces the heat susceptibility of the spores to about 60 per cent.

In order to confirm the belief that the heat-sensitizing action of the rays transmitted by fluorite was caused by the very short rays in the Schumann region the spores were exposed 5 mm. from the window. An air column of this thickness will absorb practically all the Schumann rays as pointed out by Pflüger (1904). Increasing the path of light does, of course, reduce the light intensity, but the decreased sensitization resulting therefrom is obviously much greater than can be explained by the inverse-square law. In calculations based on the inverse-square law the reduction in intensity could not exceed 12 per cent, and inasmuch as this is not a point source, the reduction in intensity is less (of the order of 4 to 6 per cent), whereas the observed reduction in heat susceptibility is from 91 per cent to 52 per cent.

The data recorded in this table represent the averages of 10 separate experiments in which the deviation from the average was approximately ± 4.5.

DISCUSSION

In this study, sensitization to heat was observed only under conditions which were sporocidal for a considerable number of cells. Sublethal exposures did not measurably decrease the heat-resistance of spores so treated. It is apparent that there is a minimum level of radiant energy below which sensitization does not occur. Where the weaker cells are concerned this threshold value may exceed their ultraviolet light tolerance; this we believe to be the most plausible explanation for the low sensitization which accompanies low mortality.

The exact mechanism by which ultraviolet light renders bacteria more susceptible to heat is a matter for conjecture. A satisfactory explanation may be found in the reactions known to occur in simple proteins under the influence of ultraviolet radiation.

Clark (1935) has demonstrated that the coagulation of isoelectric egg albumin solutions by ultraviolet radiation involves three distinct processes; first, a physical process, independent of temperature and largely independent of hydrogen-ion concentration; secondly, a chemical reaction between the light-denatured molecule and water, which is similar in some respects to denaturation by heat, and finally flocculation of the previously-altered molecules. The significant fact observed by Clark was that heat-denaturation, the precursor of flocculation, occurs at a lower temperature after light-denaturation; in the latter state, therefore, the protein must be in a chemically active condition.

By analogous reasoning, bacterial protoplasm is converted by the absorption of ultraviolet energy into a more reactive and therefore less resistant state, in consequence of which the subsequent processes of heat-denaturation and flocculation occur at lower energy levels.

The permanent nature of light-denaturation is in accord with our observation previously described in which heat sensitization effects were found to be independent of the time elapsing between irradiation and heat treatment. The irreversibility of the reaction with Paramecia appears to be open to question since Bovie and Klein (1918) observed complete recovery from the effects of Schumann rays when sufficient time elapsed before heat was applied.

SUMMARY

Many spores which survive lethal heat or ultraviolet radiation are thereby sensitized to that particular influence and in consequence are more easily killed by further applications of the same treatment than are untreated or control spores.

Many spores which survive lethal ultraviolet radiation are thereby sensitized to heat. The number of spores sensitized to heat by ultraviolet radiation is less than the number sensitized to light by ultraviolet radiation; similarly the number of spores sensitized to heat by ultraviolet radiation is less than the number sensitized to heat by heat.

Apparently not all spores are susceptible to sensitization by

heat; those moderately and highly resistant to light and heat are most affected.

When spores are exposed to the combined action of heat and ultraviolet radiation the order of treatment materially affects the number which will survive. Under conditions of adequate irradiation, the light-heat sequence is always the more destructive.

Heat has no appreciable light-sensitizing action upon spores.

Ultraviolet rays of wave lengths longer than 2,000 Å., under suitable conditions of exposure, sensitize spores to heat. Ultraviolet rays in that portion of the Schumann region between 1,250 and 1,600 Å. are more effective in sensitizing spores to heat than those transmitted by quartz.

It is a pleasure to acknowledge our indebtedness to Dr. W. W. Coblentz of the National Bureau of Standards for his counsel in regard to air-space absorption and analysis of these data, and to Dr. G. R. Greenbank of these laboratories for assistance in radiometric measurements.

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COMPARATIVE METABOLISM OF THE R AND S VARIANTS OF LACTOBACILLUS PLANTARUM (ORLA-JENSEN)

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Received for publication May 9, 1938

The extreme variability in fermentation reactions of lactobacilli is well known. Weiss and Rettger (1934), Curran, Rogers and Whittier (1933), and Howitt (1930) have ably reviewed this subject.

The alterations in fermentative reactions have seldom however been clearly correlated with colonial dissociation. Roos (1927) isolated from one strain of *Lactobacillus acidophilus* three distinct fermentative and colonial types. Hadley, Bunting and Delves (1930) found that R variants obtained from S strains of oral aciduric bacteria fermented glucose readily but differed slightly in their reactions to the S type on other sugars. R variants, designated as group III, fermented glucose and showed depressed activity with other sugars. Curran, Rogers and Whittier (1933) separated aciduric strains by correlating colony form, effect of temperature on growth, type of lactic acid produced and fermentation reaction. Kopeloff and Kopeloff (1937) observed that R variants of *L. acidophilus* produced inactive lactic acid while the S type produced *d* lactic acid. R variants of *Lactobacillus bulgaricus* developed both *d* lactic and inactive lactive acid.

The present study demonstrates a correlation of colony type and fermentative reaction of *Lactobacillus plantarum*. A marked depression of carbohydrate activity along with a definite enhancement of non-saccharolytic properties is noted in the dissociation of S to R.

ORIGIN OF CULTURE

The lactobacillus used in this study was originally isolated as the S type from olive brine (Tracy, 1934). During the several years this organism has been serially cultivated in glucose broth, it has shown enhancement of fermentative capacities. Originally it failed to ferment lactose, raffinose, arabinose and dextrin. Now, however, these carbohydrates are readily fermented.

Following the classification of Pederson (1936), this strain has been designated *Lactobacillus plantarum.* The properties exhibited by it, even to the limits of salt tolerance, closely approximate the characters presented by Dr. Pederson for the species, *L. plantarum* (Orla-Jensen).

DISSOCIATION

The most reliable method developed for the dissociation of S to R was the usual rapid serial cultivation of the S type in 1 per cent glucose broth followed by plating (streak method) on glucose agar containing 4 per cent NaCl. Dissociation in this manner appeared to be abrupt, as intermediate colonial types were seldom encountered. LiCl broth or agar failed to induce dissociation from S to R. Ageing in gelatin, glucose agar, glucose broth, serial transfer in $FeCl_3$ (0.1 per cent) broth or agar irregularly induced S to R variation.

R to S dissociation has not been accomplished. The dissociative path of this species of lactobacillus was directly opposite to that of *L. acidophilus* as shown by Upton and Kopeloff (1932) and Raney and Kopeloff (1934) who consistently failed to induce S to R variation but readily produced R to S transformation.

Stock strains of the R type were very stable, and have been subcultured continuously in 1 per cent glucose broth, or glucose broth containing 4 per cent NaCl. Stock strains of the S type were stable when subcultured once or twice weekly in glucose broth. Occasionally "accidental" ageing (2 weeks) induced dissociation, especially in mannitol and fructose broth.

FERMENTATION OF CARBOHYDRATES BY R AND S VARIANTS

Growth in glucose broth

Both R and S variants grew luxuriantly in 1 per cent glucose broth. The lag period of growth was shorter for the S type, and the maximum number of cells produced was greater by both total and viable counts. In general, the total count for the S type at 37°C. was 4 × 10^9 cells per cubic centimeter and for the R type it was 5 × 10^8 cells per cubic centimeter although not uncommonly 10^9 cells per cubic centimeter were produced.

Both R and S types settled completely after maximum growth. The S type of sediment was loose while the R type was stringy and mucus-like. Growth was similar in glucose broth containing 4 per cent NaCl, except that longer lag periods were noted, and less cells per cubic centimeter were present at maximum growth.

Microscopically, cells of the S type were evenly dispersed throughout the field, while the R type of cells, which invariably occurred in small, tight clusters or packets of 5 or 10 cells each, were unevenly distributed. This character of the R type interfered with obtaining reliable bacterial counts.

Acid production in glucose broth

Although the S variant readily produced acid when grown in glucose broth, the R variant failed to produce any titrable acid. If the R variant was incubated for a prolonged period of time, however, titrable acid would irregularly be detected in small quantities.

In table 1, data are presented comparing the acid production of R and S variants grown in 1 per cent glucose broth.[1] The data show that titrable acid is not produced by the R variant even after maximum growth is reached, although the S variant produces titrable acid as early as 12 hours after initial inoculation.

In this test, as in following ones, titrations for total acid were made on 1 cc. samples of glucose broth. The burette containing base was 10 cc. in capacity, graduated to 0.05 cc. volumes. The pH determinations were made with a Leeds and Northrup quinhydrone apparatus. Bacterial counts were made by serially diluting 1 cc. samples of culture in glucose broth, then plating on glucose agar as usual. Both the plates and dilution tubes were incubated at 37°C. for 4 to 5 days before counting. Close agreement between positive tubes and plate counts usually was obtained.

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[1] These protocols are individual tests on the R and S variants among a large number of similar experiments made for the purpose of determining maximum zymase activity. All tests have the trend shown in table 1.

An R variant, subcultured in 1 per cent glucose broth containing either 1 or 4 per cent of NaCl for 34 and 64 subcultures respectively, failed to produce titrable acid when the incubation period at 37°C. between subcultures was 50 hours or less. In table 2, data are presented showing the acid production in these strains when the incubation period between subcultures exceeded 50 hours.

TABLE 1

Production of acid by R and S variants in 1 per cent glucose broth

TIME OF FERMENTATION AT 37°C.	NUMBERS OF VIABLE CELLS PER CC.		N/10 NaOH TO NEUTRALIZE 1 CC. SAMPLE OF BROTH PHENOLPHTHALEIN INDICATOR		pH OF BROTH		TOTAL ACID IN TERMS OF LACTIC ACID PER CC. OF BROTH		ACID PRODUCED PER CC. IN TERMS OF LACTIC ACID	
	R	S	R	S	R	S	R	S	R	S
hours			*cc.*	*cc.*					*mgm.*	*mgm.*
0	10^5	10^5	0.22	0.20	6.1	6.1	1.98	1.80	0.00	0.00
4		10^6	0.21	0.20	6.3		1.89	1.80	0.00	0.00
8	10^6	10^7	0.21	0.20		6.1	1.89	1.80	0.00	0.00
12	10^7	10^8	0.20	0.23		5.8	1.80	2.07	0.00	0.27
16	10^8	10^8	0.20	0.24	6.3		1.80	2.16	0.00	0.36
20	10^8	10^9	0.24	0.30		5.2	2.16	2.70	0.18	0.90
22	10^9	10^9	0.21	0.32		4.9	1.89	2.88	0.00	1.08
24	10^9	10^9	0.21	0.33	6.1	4.9	1.89	3.07	0.01	1.27
30	10^9	10^9	0.20	0.36	6.5	4.7	1.80	3.24	0.00	1.44

The values show that acid may be produced by the R variants when prolonged incubation periods in glucose broth are maintained. Nevertheless, the quantity of acid produced in this period is equivalent only to that formed by the S variant in 12 to 20 hours. Only R variants have been isolated from these acid-forming R cultures.

In table 3, data are presented to show that the S variant readily uses glucose simultaneously with acid production, while the R variant fails to utilize glucose and is unable to form acid. The Schaffer-Hartmann method was used to determine the presence

TABLE 2

Acid production by R variant continuously subcultured in glucose broth

TIME OF INCUBATION AT 37°C.	DIFFERENCE IN INITIAL AND FINAL TITRATIONS OF N/10 NaOH				TOTAL ACID PRODUCED IN TERMS OF LACTIC ACID PER CC.	
	1% glucose broth				1% glucose broth	
	1% NaCl		4% NaCl		1% NaCl	4% NaCl
	R	Control	R	Control	R	R
hours	*cc.*	*cc.*	*cc.*	*cc.*	*mgm.*	*mgm.*
53	0.01	0.02	0.00	−0.02*	0.00	0.00
67	0.01	−0.04	0.00	−0.03	0.00	0.00
76	0.03	−0.03	0.00	0.01		0.00
94	−0.02	0.04	0.02	−0.01	0.00	
99	0.01	0.01	0.00	−0.03	0.00	0.00
days						
8	−0.03	0.04	0.02	0.04	0.00	0.00
8.5	0.04	0.01	0.01	−0.03	0.27	
9	−0.03	0.03	0.00	0.01	0.00	0.00
10	0.08	0.02	0.13	0.04	0.54	0.81
11.5	0.06	0.06	0.09	0.04	0.00	0.45
26	0.24	0.20	0.18	0.13	0.36	0.45

* Final titration more alkaline than initial titration by −0.02 cc. Average experimental error in titration ±0.05 cc.

TABLE 3

Utilization of glucose by R and S variants grown in 1 per cent glucose broth

	R VARIANT							S VARIANT						
	Time of incubation an 37°C.													
	0 hours			24 hours			8 days	0 hours			24 hours			8 days
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 3	Test 4	Test 5	Test 6	Test 4	Test 5	Test 6	Test 6
Total acid in terms of mgm. of lactic acid per cc.	1.98	1.98	2.3	1.98	1.8	2.3	3.0	1.80	1.80	1.80	3.87	3.0	4.1	8.2
Reducing substances in terms of mgm. of glucose per cc.	10.92	10.92	12.60	10.92	10.87	12.90	13.10	10.00	10.50	12.80	8.57	9.38	10.10	5.2
Mgm. of glucose utilized per cc.				0.00	0.05	0.00	0.00				1.43	1.12	2.70	7.60
Mgm. of acid formed per cc. in terms of mgm. of lactic acid				0.00	0.00	0.00	0.07				2.07	1.20	2.3	6.4

of reducing substances in the culture media. In most tests, proteins were not precipitated from the culture media before testing for reducing substances.

In table 4, data are given which show that lactic acid is formed by the S variant but is not formed by the R variant. The method of Friedmann, Cotonio, and Schaffer, as modified by Wendel (1935) was used to test for the presence of lactic acid.[2]

Acid production in different carbohydrates

When the R and S variants were grown in broth containing carbohydrates other than glucose, titrable acid was formed by the S type but not by the R variant. Data are given in table 5.

TABLE 4

Production of lactic acid by R and S variants in 1 per cent glucose broth

	TIME OF INCUBATION AT 37°C.				
	24 hours		Broth control	48 hours	
	R variant	S variant		R variant	S variant
Total titrable acid produced in terms of mgm. of lactic acid per cc.	0.09	3.78	0.00	0.09	4.86
Mgm. of lactic acid per cc.		5.9	2.7	2.3	6.4
		6.0	3.0	2.5	6.8
Mgm. of lactic acid produced per cc.		3.10		0.00	3.75

These results indicate that the R variant is generally devoid of active carbohydrases. Growth of the S variant in each of these media was luxuriant and produced approximately equal turbidity in the different compounds; the R variant, however, grew sparingly in each medium. The S variant had been continuously subcultured in glucose broth since initial isolation. The glucose R variant had been subcultured serially in glucose broth for 20 transplants, while the sugar-free R variant had been carried in sugar-free broth for 49 transfers. Sugar-free broth was prepared by mixing 1 per cent each of beef extract (Difco), NaCl, and sugar-free peptone in distilled water. Sugar-free peptone was prepared by the usual method of incubating, at 37°C., heavy suspensions of *Escherichia coli* in 10 per cent peptone solutions for 48 to 72 hours under vaseline seal.

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[2] Mr. Charles Lombard, chemist, Los Angeles County Hospital, directed and generously aided in these lactic acid determinations.

FERMENTATION OF CARBOHYDRATES BY "RESTING" CELL SUSPENSIONS

In order to exclude the possibility that insufficient concentration of cells in the R type of culture prevented the development of acid, the production of acid by "resting" cell suspensions was tested. R and S variants were grown in broth containing 1 per cent carbohydrate, centrifuged and "washed" in saline 3 times. Arabinose, glucose, lactose and raffinose were the carbohydrates used except in the case of the R variant which was grown only in glucose broth. These cells were added to solutions of various carbohydrates in 0.01 M phosphate buffer and 0.01 M phosphate buffer plus 1 per cent peptone so that the final concentration was 10 mgm. of cells (wet weight) per cubic centimeter. The initial pH in each tube was 6.2. The results of this test are presented in table 6.

TABLE 5

*Production of titrable acid by R and S variants grown in various carbohydrate broths**

VARIANT GROWN IN SPECIFIC CARBOHYDRATE	TIME OF INCUBATION AT 37°C.	ARABINOSE	FRUCTOSE	MANNOSE	GALACTOSE	SUCROSE	LACTOSE	RAFFINOSE	GLYCEROL†
		Total acid in terms N/10 NaOH in cc.							
	hours								
Glucose S . . .	72	0.15	0.61	0.48	0.59	0.27	0.12	0.18	0.11
Glucose R .	72	0.00	−0.05	0.00	0.01	0.01	−0.05	0.03	−0.04‡
Sugar-free R	72	0.00	0.02	0.02	0.03	0.03	−0.03	−0.02	−0.03

* Nutrient broth containing 1 per cent of a given carbohydrate. Experimental error in titration = ±0.05 cc.

† Included as the three carbon compound since glycerose was unavailable.

‡ Final titration more alkaline than initial titration by 0.04 cc. N/10 NaOH.

Although the average experimental error in titrating was somewhat large (±0.07), the values clearly indicate that specific carbohydrases were developed by the S variant for the particular substrate in which it had been grown except in the case of glucose. Interpretation of the data in accordance with Karstrom (1930), suggests that zymase of the S variant is constitutive in character while the enzymes attacking arabinose, lactose and raffinose are adaptive. The R variant failed to produce titrable acid in any of the carbohydrates tested. Although the titrable acid was greater in the carbohydrate solutions containing peptone in nearly every case with the S variant, the R form was unaffected by peptone.

TABLE 6

Fermentation of various carbohydrates by R and S variants grown in specific sugars

VARIANT GROWN IN SPECIFIC CARBOHYDRATE	FERMENTATION AT 37°C.	TOTAL ACID IN TERMS OF N/10 NaOH							
		Phosphate buffer				Phosphate buffer + 1 per cent peptone			
		Arabinose	Glucose	Lactose	Raffinose	Arabinose	Glucose	Lactose	Raffinose
	hours	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Arabinose S	2	0.10	0.10	0.00	0.00	0.24	0.24	0.04	0.00
	5	0.17	0.20	0.03	0.00	0.30	0.35	0.06	0.04
Glucose S	2	0.02	0.07	0.00	0.00	0.01	0.12	0.03	0.01
	5	0.02	0.10	0.02	0.03	0.05	0.16	0.04	0.03
Lactose S	2	0.00	0.12	0.06	0.00	0.03	0.11	0.11	0.05
	5	0.00	0.16	0.30	0.03	0.02	0.26	0.21	0.07
Raffinose S	2	0.07	0.12	0.06	0.10	0.00	0.20	0.10	0.22
	5	0.03	0.26	0.19	0.14	0.02	0.26	0.27	0.37
Glucose R	2	0.00	0.03	0.00	0.06	0.00	0.01	0.03	0.00
	6	0.00	0.00	0.00	0.00	0.00	0.06	0.07	0.03
Control titration without bacteria	2	0.00	−0.03	−0.04	−0.02	−0.01	−0.07	0.00	0.03
	5	0.00	0.00	0.00	−0.03	−0.04	−0.05	−0.01	0.03
	6	−0.02*	−0.06	−0.03	0.03	−0.04	0.07	−0.02	0.00

* Final titration was more alkaline than initial titration by 0.02 cc. N/10 NaOH. Experimental error ±0.07.

GROWTH OF R AND S VARIANTS IN CARBOHYDRATE-FREE MEDIA

That lactobacilli have a distinct saccharolytic type of metabolism has been well known since Rahe (1914 and 1918) emphasized that aciduric bacteria gave luxuriant growth in unneutralized meat-peptone broth only when suitable carbohydrates were added. Weaver (1932) noted that *L. acidophilus* and *L. bulgaricus* did not grow in sugar-free basic media. However, proteolytic activities of lactobacilli have been noted by Gorini (1922), Kendall and Haner (1924a, b), Kulp and Rettger (1924), Peterson, Pruess and Fred (1928), and Frazier and Rupp (1931).

When the R and S variants were cultured in nutrient broth, the R variant readily grew but the S variant developed only sparingly. Serial cultivation of the S type was possible usually for three transfers in this medium; the R variant, however, could be transferred continuously without difficulty. When these strains were cultured in sugar-free broth, the S form failed to grow but the R variant readily developed. In each of these tests, the inocula were "washed" cell suspensions grown in glucose broth. The initial concentration of cells in the test media approximated 10^7 cells per cubic centimeter although 10^5 cells per cubic centimeter were often used for the R type.

In table 7, growth of the variants in different media is summarized. The data show that not only will the R form grow in sugar-free peptone solutions while the S type fails to do so, but also that the R variant can grow in a smaller concentration of peptone than the S form even when glucose is available.

Further behavior of these variants is shown in table 8. The phosphotungstic acid precipitate of an acid alcohol extract of peptone was prepared according to the method of Sahyun, *et al.* (1936). Butyl alcohol fractions of the extract were not prepared. The casein was dissolved in N/10 NaOH, diluted, and neutralized to pH 7 with weak HCl. The data show that the R variant grows in the alcoholic extract of peptone and in casein in the absence of glucose while the S form grows in the extract only when glucose is present. The S form does not grow in the casein medium even in the presence of glucose. These results suggest that the R variant possesses a greater proteolytic activity than the S type. However, as growth of the R variant in casein was slow and extremely slight, it is possible that only hydrolysis products of casein, formed on dissolution in alkali, acted as nutrients in this medium. This is in accord with the observation that the R variant requires a smaller concentration of peptone for growth than the S variant.

TABLE 7

Comparison of growth of R and S variants in sugar-free and sugar-containing media[*]

MEDIUM	S VARIANT		R VARIANT	
	Time of incubation at 37°C.	Growth	Time of incubation at 37°C.	Growth
	days		*days*	
Sugar-free broth	7	−	2	++++
Sugar-free broth + 1 per cent glucose	2	++++	2	++++
Sugar-free peptone[†] in per cent:				
1.0	7	−	2	++++
0.1	7	−	7	+++
0.075	7	−	7	Trace
0.05	7	−	7	−; +[‡]
Sugar-free peptone + 1 per cent glucose in per cent of peptone:				
1.0	2	++++	2	++++
0.1	7	+	7	+++
0.075	7	−	7	+
0.05	7	−	7	−; +

[*] Growth is recorded according to the turbidity developed in the medium. No attempt was made to compare quantitatively the growth of the R and S variants.

[†] This medium was prepared by diluting sugar-free peptone in a basal salt solution of the composition in per cent as follows: K_2HPO_4, 0.17; KH_2PO_4, 0.14; $MgSO_4\cdot 7H_2O$, 0.02; $CaCl_2$, 0.01; NaCl, 0.5.

[‡] −; + indicates that in some tests growth occurred although in the majority of tests no growth was noted.

Nevertheless, these tests with carbohydrate-free media indicate that a marked difference in the metabolism of the two variants exists. This difference is especially pronounced when the utilization of carbohydrates is considered. The observations indicate that the S variant possesses a saccharolytic metabolism with little or no ability to utilize nitrogenous compounds in the absence of carbohydrates while the R variant possesses a nitrogenous metabolism devoid of saccharolytic activities. Although the latter retains or gains some ability to utilize glucose as is evident after prolonged incubation in glucose broth, this behavior is not significant from the viewpoint of being essential for growth.

TABLE 8

Growth of R and S variants in alcoholic extract of peptone and in casein solutions

MEDIA*	CONCENTRATION OF N_2 COMPOUNDS	TIME OF INCUBATION AT 37°C.	GROWTH OF VARIANTS†	
			R	S
	mgm. per cc.	*days*		
Extract	10.0	4	++++	−
	1.0	4	++	−
	0.1	5	+	−
Extract + glucose (1 per cent)	10.0	4	++++	++++
	1.0	4	++	++
	0.1	5	±	−
Casein‡	10.0	5	+	−
Casein + glucose (1 per cent)	10.0	5	+	−
Casein + glucose (1 per cent) + peptone (1 per cent)	10.0	2	++++	++++

* Prepared by dissolving the alcoholic extract of peptone and the casein in basal salts solution.

† Inocula were cells cultured in glucose broth for 24 hours, and washed once in saline. Initial concentration of cells in test media approximated 10^7 per cc.

‡ "Labco" purified, vitamin-free casein.

Of interest in this connection are the studies of both Hoogerheide (1937) who obtained from a non-saccharolytic strain of *Clostridium histolyticum*, a saccharolytic variant similar to *Clostridium sporogenes*, and Goodman (1908) who developed a non-acid producing strain of *Corynebacterium diphtheriae* from a normal toxogenic stock culture. It is concluded that by the dissociation of *L. plantarum* from S to R, a saccharolytic bacterium is changed to a non-saccharolytic type possessing enhanced capacities for nitrogenous nutrients.

SUMMARY AND CONCLUSIONS

1. It was found that rapidly growing cultures of *Lactobacillus plantarum* would dissociate from S to R when plated on glucose agar containing 4 per cent NaCl.
2. The R variant failed to produce titrable acid when grown for 1 to 8 days at 37°C. in 1 per cent glucose broth, although the S variant rapidly produced acid in this medium. The R variant produced detectable quantities of acid in glucose broth, however, after prolonged incubation periods (8 to 26 days).
3. The R variant did not utilize glucose when grown in glucose broth at 37°C. for 8 days, although the S variant utilized approximately 60 per cent of the available glucose in this medium in 8 days.
4. Lactic acid was produced by the S variant but was not formed by the R variant.
5. Although the R variant was grown at 37°C. for 72 hours in broth containing carbohydrates other than glucose, titrable acid was not produced. The S variant readily fermented these carbohydrates.
6. "Resting" cell suspensions of the R variant consisting of approximately 10 mgm. of cells per cubic centimeter failed to form titrable acid in arabinose, glucose, lactose and raffinose. Similar suspensions of the S variant produced acid with these carbohydrates.
7. It was found that the R variant grew in sugar-free peptone solutions or broth. The S variant failed to grow in these media in the absence of carbohydrates.
8. The R variant but not the S type grew in carbohydrate-free solutions of casein or an alcoholic extract of peptone. The S variant grew in the alcoholic extract of peptone when glucose was present but failed to grow in casein medium containing glucose.
9. It is concluded that in the dissociation of *Lactobacillus plantarum* from S to R, a saccharolytic bacterium is changed into a non-saccharolytic type possessing enhanced capacities for nitrogenous nutrients.

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THE BACTERICIDAL EFFECT OF SULFANILAMIDE UPON BETA HEMOLYTIC STREPTOCOCCI IN VITRO

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Received for publication May 11, 1938

The therapeutic efficacy of sulfanilamide in clinical and experimental infections due to Beta-hemolytic streptococci and certain other bacteria seems to be established. However, the mechanism underlying the action of the drug remains open to conjecture.

Levaditi and Vaisman (1935) suggested that the effectiveness of the drug is due to interference with formation of bacterial capsules. Neither Colebrook and his associates (1936) nor Long and Bliss (1937a) were able to confirm this.

Attention was focussed upon the possible effect of the drug on phagocytosis, when Long and Bliss (1937a) found an unusual increase in phagocytosis of streptococci by both polymorphonuclear and mononuclear leucocytes in peritoneal smears from mice recovering under sulfanilamide therapy. Mellon, Gross and Cooper (1937) studied histological sections of subcutaneous streptococcus lesions in treated and untreated guinea pigs, and were unable to demonstrate that phagocytosis played a primary role in the curative process. Weinberg, Mellon, and Shinn (1937) in work with several hundred mice, found no clear evidence of increased phagocytosis by leucocytes or reticulo-endothelial cells of liver or spleen in drug-treated animals. Long has recently been quoted by Bohlman (1937) as believing that phagocytosis is neither inhibited nor enhanced by the drug.

The possibility of a direct effect of the drug upon bacteria has attracted considerable attention. Colebrook and his associates (1936) and Long and Bliss (1937 a and b; also Bliss and Long, 1937) have reported a bacteriostatic effect upon streptococci with sulfanilamide in culture media. The failure of Rosenthal (1937) to confirm this was probably due to use of large inocula. The easily demonstrated bacteriostatic action of the drug *in vitro,* together with the findings of Bliss and Long (1937) in connection with experimental infections in mice, seems to throw the weight of evidence in favor of the theory that the action is directly upon the bacteria themselves rather than an effect upon the usual elements involved in defense.

If we accept the view that sulfanilamide acts directly upon bacteria, the question arises as to whether this action is inhibitory or bactericidal (in the sense of sterilization). Helmholtz and his associates (1937), among others, have reported a bactericidal effect with the drug in certain infections of the urinary tract. This effect in urine has been demonstrated *in vitro.* A bactericidal effect in blood has been demonstrated by Colebrook and his associates (1936). This has been confirmed by Finklestone-Sayliss (1937) who found that a bactericidal effect was obtained in de-leucocyted blood as well as in whole blood. Osgood and Brownlee (1938) have reported that sulfanilamide in human marrow cultures effects sterilization when streptococci are added, but these investigators do not believe that the drug alone is able to kill the organisms. Gay and Clark (1937) state that the drug "has no bactericidal effect on the streptococcus when added to nutrient media in the test tube." Bliss and Long (1937) have reported experiments which do not support the belief that sulfanilamide has a bactericidal effect *in vitro.* Nitti and his associates (1937), and Mayer (1937), on the other hand, reported experiments in which the drug apparently brought about sterilization in peptone-glucose water and saline, respectively, when small inocula of streptococci were used.

It is evident from the literature cited above that the question as to whether sulfanilamide acts as a bacteriostatic or as a bactericidal agent is still unsettled.

Although test temperatures were not mentioned in every case, it is probable that the above-mentioned investigations on the effectiveness of the drug *in vitro* have been carried out at approximately 37°C. As far as we have been able to determine, the effect of the drug at higher temperatures upon streptococci *in vitro* seems to have been overlooked.

The present report contains preliminary data bearing on the bactericidal action of sulfanilamide upon Beta-hemolytic streptococci *in vitro* at an elevated test temperature. Experiments designed to evaluate the factors which condition this bactericidal action will form the basis of subsequent reports.

MATERIALS AND METHODS

Strains. With the exception of B-168, all strains used in the present study had been recently isolated from cases at the Childrens Hospital.[1] These cases included septicemia, meningitis, mastoid, otitis media, peritonitis and scarlet fever infections. Colony variants, determined in accordance with the criteria suggested by Ward and Lyons (1935), included F, M, C and a fourth variant, not as yet described, which we have tentatively designated as the *B variant.* B-168 is the "Richards" strain, used by Colebrook and his associates (1936) and provided through the courtesy of Dr. R. C. Lancefield. Several of the strains used in our tests were highly virulent for mice. The B and M variants developed thick capsules in the PD broth described below.

Stock cultures. Cultures in defibrinated horse blood were stored at refrigerator temperature and transferred to fresh horse blood once a month. Under these conditions all four colony variants were found to be stable.

Test cultures. Frequent transfer in PD broth was maintained for all cultures tested. The test inoculum consisted of 0.2 cc. of a 1:1000 dilution in broth of a 3- to 5-hour broth culture grown at 37°C. An average initial concentration yielding 5000 colonies per cubic centimeter of test mixture was thus obtained, as determined by blood-agar plate counts.

[1] We wish to thank Dr. Benjamin Carey for his courtesy in supplying the Childrens Hospital strains.

Sulfanilamide solutions. Pure para-amino-benzene-sulfonamide, supplied by the Winthrop Chemical Company, was used in our tests. A 0.44 per cent stock solution in distilled water, kept away from light, gave consistent results in repeated tests. 0.2 cc. of stock solution was used to obtain an initial concentration of 20 milligrams per cent in test mixtures. No change in this concentration of drug was detected on completion of tests.

Peptone-Glucose medium (PD broth)

Neopeptone	7.0 grams
Proteose peptone	7.0 grams
Pfanstiehl peptone	7.0 grams
Sodium chloride	5.0 grams
Potassium phosphate, primary	1.0 grams
Potassium phosphate, secondary	1.0 grams
20 per cent sodium hydroxide	3.8 cc.
Distilled water	1000 cc.

Heat ingredients listed above at 100°C. for 20 minutes. Filter. Add 1.0 gram of glucose, dispense and autoclave for 20 minutes at 112°C. The resulting clear buffered broth should have a pH of approximately 7.5. Abundant and rapid growth from small inocula of many different strains of Beta-hemolytic streptococci has been obtained in this broth. The buffer system prevents the pH from dropping below 6.0 during maximum growth and, thus, death of organisms in old cultures is delayed. Many strains develop capsules in young cultures in this medium. Several strains highly virulent for mice have retained their virulence when cultured in it. PD medium was used throughout the present study: as plain broth; as blood broth, with one drop of defibrinated horse blood added to 9 cc. of PD; as plating medium, with the addition of 1.5 per cent agar.

Bactericidal test procedure. Test mixtures were made in round-bottomed rubber-stoppered flasks (25 cc. capacity) in pairs for each strain as follows:

	Flask A *cc.*	*Flask B (Control)* *cc.*
Medium (Blood or Broth)	4.0	4.0
Sulfanilamide 0.44 per cent	0.2	
Saline 0.85 per cent		0.2
Culture diluted 1:1000	0.2	0.2

The flasks were attached in a horizontal position to a wheel which was revolved at 10 R.P.M. in an incubator. As each flask rotated, its contents were continuously spread and rolled over the glass surface, which insured satisfactory mixture throughout the test period. The initial concentration of each test mixture was determined by making a similar mixture and immediately transferring 1.0 cc. through a series of 9 cc. blood broth dilution blanks. The bacterial concentration was expressed as the logarithm_{10} of the highest dilution of test mixture in blood broth which showed growth after 72 hours incubation at 37°C. After incubation of mixtures for test period, bacterial concentrations were again determined by quantitative dilution in tubes of blood broth. Growth in blood broth tubes was checked by loop streaks on blood agar plates.

Criteria for sterility in test mixtures. Test mixtures were considered to be sterile when growth was not obtained in the following subcultures incubated at 37°C. for 72 hours: (*a*) loopful streaked on blood agar plate; (*b*) blood agar pour plate culture of 0.5 cc. of undiluted test mixture; (*c*) 1.0 cc. of test mixture added to 9 cc. of PD blood broth.

RESULTS

Preliminary tests had convinced us that a concentration of as much as 100 mgm. per cent sulfanilamide in whole blood, or broth, at 37°C. failed to sterilize an inoculum of certain strains of Beta-hemolytic streptococci; when an initial concentration in test mixtures of 1,000 to 10,000 bacteria per cubic centimeter was used.

During the course of some work carried out at the Harvard School of Public Health, a year ago, it was observed that many freshly isolated strains of hemolytic streptococci grew rapidly and abundantly at 40°C. A few trial mixtures of sulfanilamide and streptococci in blood were tested at elevated temperatures and what appeared to be a bactericidal action, due to the drug, was consistently obtained at 40°C. with 20 mgm. per cent sulfanilamide in horse blood during 48 hours incubation. The bactericidal effect was not obtained at 37°C. These observations led to the present study.

The first part of our work was directed to determine if the bactericidal action noted above was confined to the particular strains used. Accordingly, the bactericidal test technique, described elsewhere in this paper, was used with 36 different strains freshly isolated from various human infections. In all cases, sterilization of the test inoculum, in normal defibrinated horse blood containing 20 mgm. per cent of the drug, was obtained during 48 hours incubation at 40°C. Control mixtures, without the drug, simultaneously incubated, showed complete hemolysis of blood and abundant growth of bacteria at the end of the test period. Blood freshly drawn gave the same result as that which had been refrigerated for as long as two weeks.

We have fully appreciated the fact that it is often extremely difficult to distinguish inhibitory and bactericidal action. Subcultures may fail to show growth, not because test mixtures are sterile, but because of continuing inhibition. The strongest concentration of sulfanilamide used in our tests was 20 mgm. per cent. Subcultures made by transferring 1.0 cc. of test mixture into 9 cc. of PD blood broth diluted the test concentration of drug 1:10, or, to 2 mgm. per cent. Therefore, tests were made to determine whether 2, or more, mgm. per cent of sulfanilamide would inhibit a small number of viable bacteria under subculture conditions, i.e., in blood broth incubated at 37°C. To one series of PD blood broth blanks sulfanilamide was added to a final concentration of 2.5 mgm. per cent. A culture of streptococci was then diluted in PD broth out to 10^{-8}. The sulfanilamide-blood-broth and a control blood-broth series were then inoculated with the culture dilutions containing decreasing numbers of bacteria and all tubes were incubated at 37°C. for 48 hours. In no case did 2.5 mgm. per cent sulfanilamide prevent abundant growth from an inoculum yielding a single colony, as determined by plate counts. Similar tests were made with several different strains. In all cases, the smallest bacterial inoculum grew out equally well, with or without the drug. Moreover, it was found that as much as 20 mgm. per cent sulfanilamide did not prevent the growth of small inocula in PD blood broth at 37°C. Additional evidence was obtained by re-inoculating test subculture tubes which appeared to be sterile with a small number of viable bacteria (from 1 to 10 colonies). The growth resulting from this second inoculum was interpreted as meaning that growth would also have resulted from the test mixture inoculum if it had contained any viable bacteria. We believe that the foregoing results justify our assumption that failure to obtain subculture growth meant sterilization, not merely inhibition, under our test conditions. Therefore, we have applied the term "bactericidal" to the effect obtained with sulfanilamide in our tests.

TABLE 1

Effect of 20 mgm. per cent sulfanilamide upon Beta-hemolytic streptococci in normal defibrinated horse blood

STRAIN	INITIAL BACTERIAL CONCENTRATION	BACTERIAL CONCENTRATION AFTER 48 HOURS			
		Sulfanilamide-blood		Blood	
		40°C.	37°C.	40°C.	37°C.
F-20	4	Sterile	7	8	9
F-24	4	Sterile		7	
F-112	4	Sterile		7	
F-115	4	Sterile		9	
M-1	4	Sterile		8	
M-146	4	Sterile	6	7	8
M-210	3	Sterile		8	
B-4	4	Sterile		9	
B-9	4	Sterile		8	
B-168	4	Sterile		8	
B-171	4	Sterile	6	8	9
B-208	4	Sterile		9	
C-17	3	Sterile		8	
C-201	4	Sterile	8	8	9
C-241	5	Sterile		7	
C-246	3	Sterile		8	

Bacterial concentrations were determined by diluting 1.0 cc. of each test mixture with blood broth 1:10, 1:100, 1:1000, etc. Figures above indicate the logarithm$_{10}$ of the highest dilution of test mixture in blood broth in which growth occurred during incubation at 37°C. for 72 hours.

The results of bactericidal tests with 16 strains are given in table 1. Bacterial concentrations in test mixtures are expressed as the logarithm$_{10}$ of the highest dilution in blood broth showing growth after 72 hours incubation at 37°C. Occasional plate counts showed that we were using an initial concentration of bacteria yielding about 5,000 colonies per cubic centimeter. In blood without sulfanilamide this initial concentration usually increased, during incubation at 40°C., to about 100,000,000 per cc., i.e., a concentration such that 1.0 cc. could be diluted to 10^{-8} and still show growth by our dilution method of subculture. Blood-bacteria mixtures to which sulfanilamide had been added, showed no hemolysis and apparently contained no viable bacteria, as determined by application of our criteria for sterility. It will be seen from the results recorded in table 1 that 20 mgm. per cent sulfanilamide was bactericidal at 40°C. for all 16 strains tested, and that this effect was not obtained with 4 strains similarly tested at 37°C.

The action of the drug in plain PD broth was then tested. The same bactericidal test method was used. The results, as recorded in table 2, indicate bactericidal action at 40°C., an effect which was lacking at the lower temperature. Four strains were used in these tests: F-20, four times; M-146, once; B-171, five times; C-201, twice.

The experiments outlined above showed that a bactericidal effect could be demonstrated with 20 mgm. per cent sulfanilamide under conditions which were otherwise favorable for abundant bacterial growth. However, the method used failed to illuminate the nature of the bactericidal action involved. Therefore, growth curves were plotted to determine the beginning and progress of bactericidal action to the point of sterility.

Growth curve procedure. Mixtures of bacteria and drug in PD broth, similar to those described above, were similarly incubated. 0.2 cc. samples removed at frequent intervals were diluted 1:10 in 1.8 cc. of broth, further quantitative dilution being carried out by transferring 1.0 cc. of this 1:10 dilution through 9 cc. blood broth blanks. 1.0 cc. of at least two dilutions was plated out in blood-agar pour plates.

Results of preliminary growth-curve tests with a large number of mixtures confirmed the observations previously noted. An inhibitory effect, due to sulfanilamide, on all strains tested, was obtained at 36°C. As the test temperature was raised to 39°C., this inhibitory effect increased. Not until the test temperature exceeded 39°C. was a bactericidal effect (sterilization) obtained with the inoculum used.

In table 3 are recorded experiments which show the effect of 20 mgm. per cent of sulfanilamide upon the multiplication of four strains of streptococci in PD broth at 37° and 40°C. In these tests 0.2 cc. of a 1:1000 broth dilution of a 4- to 6-hour broth culture was added to 4.2 cc. of broth containing enough sulfanilamide to give a final drug concentration of 20 mgm. per cent. A control flask containing no drug was similarly inoculated. All flasks were rotated to maintain conditions under which our previous results had been obtained. Samples removed at intervals of three hours were plated in blood agar, after suitable dilution. In table 3 plate counts are expressed as the

TABLE 2

Effect of 20 mgm. per cent sulfanilamide upon Beta-hemolytic streptococci in PD broth

STRAIN	INITIAL BACTERIAL CONCENTRATION	BACTERIAL CONCENTRATION AFTER 48 HOURS			
		Sulfanilamide-broth		Broth	
		40°C.	37°C.	40°C.	37°C.
F-20	4	Sterile	6	9	9
F-20	4	Sterile		9	
F-20	5	Sterile		9	
F-20	4	Sterile		9	
M-146	4	Sterile	6	8	8
B-171	6	Sterile		9	
B-171	4	Sterile	7	9	8
B-171	3	Sterile		8	
B-171	5	Sterile		8	
B-171	3	Sterile		8	
C-201	5	Sterile		8	
C-201	4	Sterile	7	8	8

Bacterial concentrations were determined by diluting 1.0 cc. of each test mixture with blood broth 1:10, 1:100, 1:1000, etc. Figures above indicate the logarithm$_{10}$ of the highest dilution of test mixture in blood broth in which growth occurred during incubation at 37°C. for 72 hours.

logarithm₁₀. These results are presented as time-action curves in charts 1, 2, 3, and 4.

The curves in chart 1 show that growth in the plain broth controls was characterized by multiplication at a rapid rate during the first twelve hours, reaching a growth level which remained practically unchanged up to the thirtieth hour of incubation. Raising the test temperature to 40°C. slowed down the optimal rate of multiplication in plain broth controls. Also, with 3 of the 4 strains, the maximal bacterial concentration at this temperature was at a level slightly lower than that reached at 37°C., as may be seen by comparing corresponding curves in charts 1 and 2. The absence of a significant lag period in any

TABLE 3

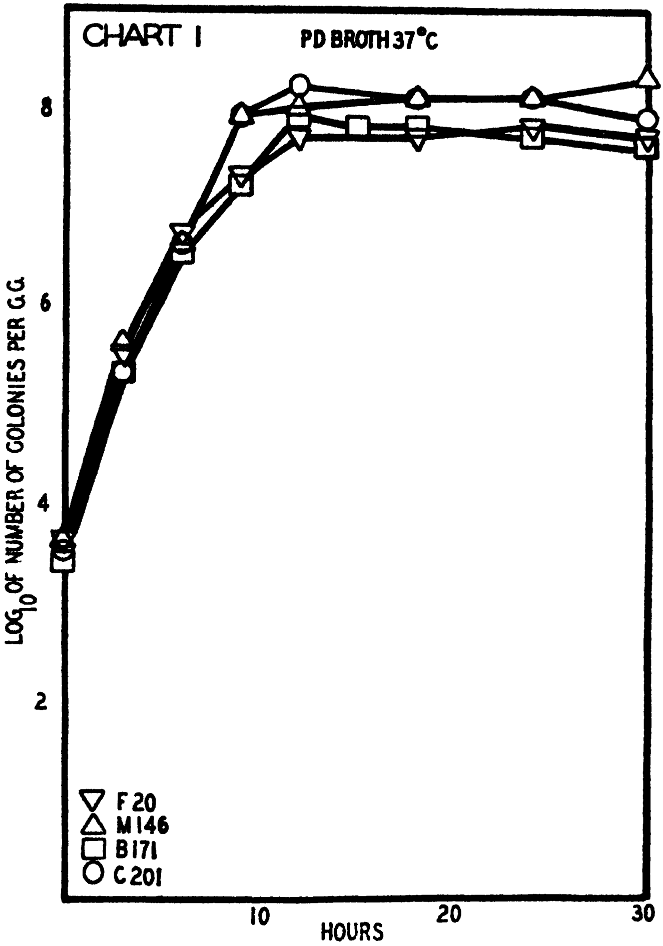
Effect of 20 mgm. per cent sulfanilamide upon the multiplication of Beta-hemolytic streptococci in PD broth at 37 and at 40°C.

STRAIN	MEDIUM	TEMPERATURE	HOURS										
			0	3	6	9	12	15	18	21	24	27	30
		°C.											
F-20	Sulf.-Broth	37	3.6	5.3	6.1	6.5	6.8		6.7		6.3		5.6
	Broth	37	3.6	5.5	6.7	7.3	7.7		7.7		7.8		7.7
	Sulf.-Broth	40	3.7	4.9	5.5	5.5		4.5	3.8		2.6		Sterile
	Broth	40	3.7	5.1	5.9	6 9		7.5	7.8		7.8		7.4
M-146	Sulf.-Broth	37	3.6	5.3	5.9	6.2	6.0		5.9		5.9		6.2
	Broth	37	3.6	5.6	6.6	7.9	8.0		8.1		8.1		8.3
	Sulf.-Broth	40	3.8	4.8	5.1	5.3		5.1	4.6		1.9		Sterile
	Broth	40	3.8	4.9	5.9	6.8		7.8	7.8		7.8		7.8
B-171	Sulf.-Broth	37	3.4	5.0	5.7	6.2	6.6	7.0	7.0		7.0		6.9
	Broth	37	3.4	5.3	6.5	7.2	7.9	7.8	7.8		7.7		7.6
	Sulf.-Broth	40	3.8	4.3	4.4	4.1	2.3	1.0	0		0		Sterile
	Broth	40	3.8	4.3	5.7	6.9	7.8	7.8	7.6		7.5		7.3
C-201	Sulf.-Broth	37	3.5	5.2	6.1	6.3	6.5		6.6		7.0		7.0
	Broth	37	3.5	5.3	6.6	7.9	8.2		8.1		8.1		7.9
	Sulf.-Broth	40	3.7	4.4	5.0	5.1	4.1	3.0	0		0		Sterile
	Broth	40	3.7	4.5	5.9	6.8	7.4	7.4	7.6		7.4		7.2

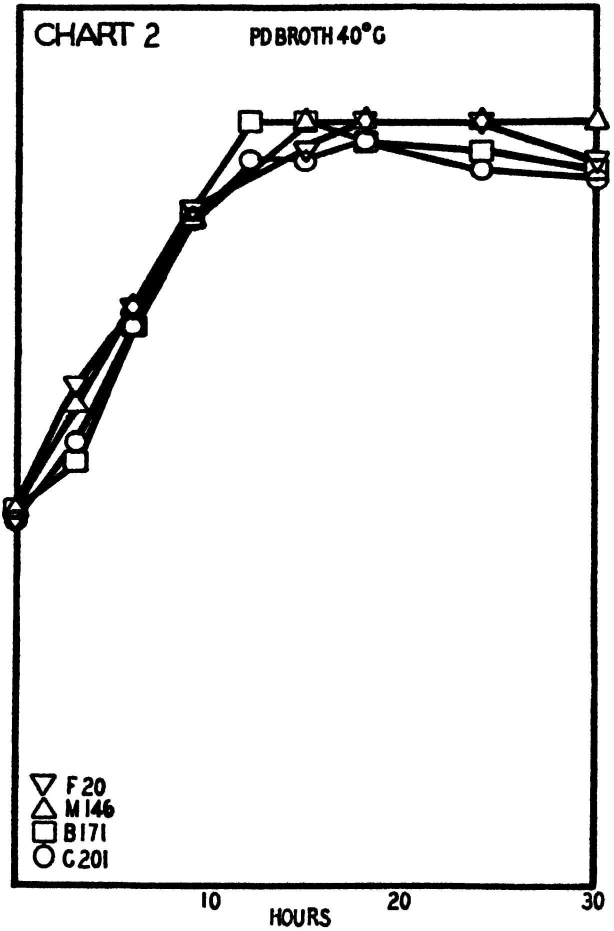
Figures in table above indicate bacterial concentrations per cubic centimeter expressed as the logarithm₁₀ of pour plate counts.

of the test mixtures is probably due to the fact that young cultures were always used as a source of test inocula.

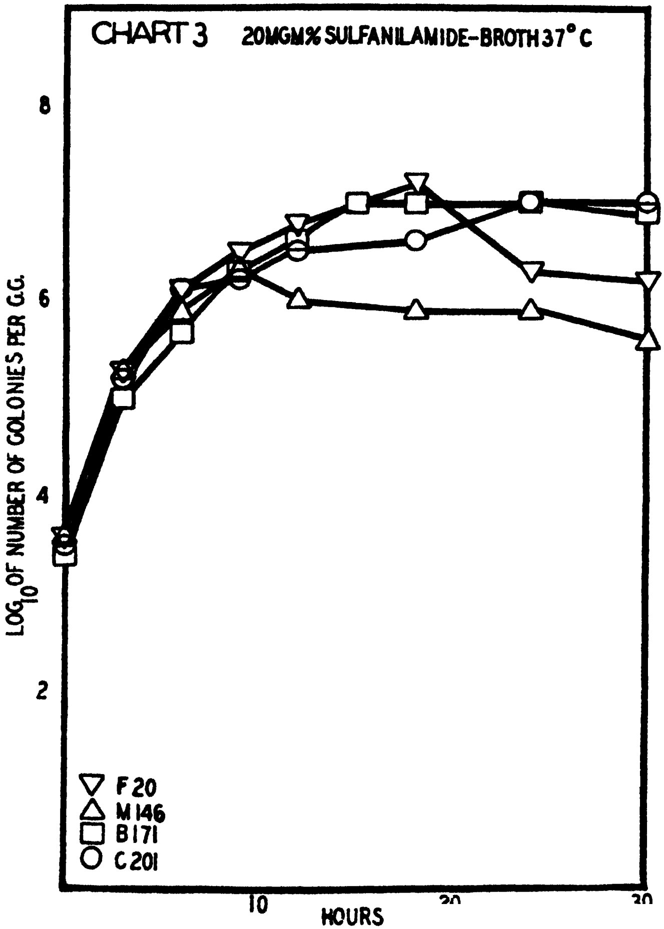
Charts 3 and 4 present growth curves obtained when bacteria were added to broth containing 20 mgm. per cent sulfanilamide,



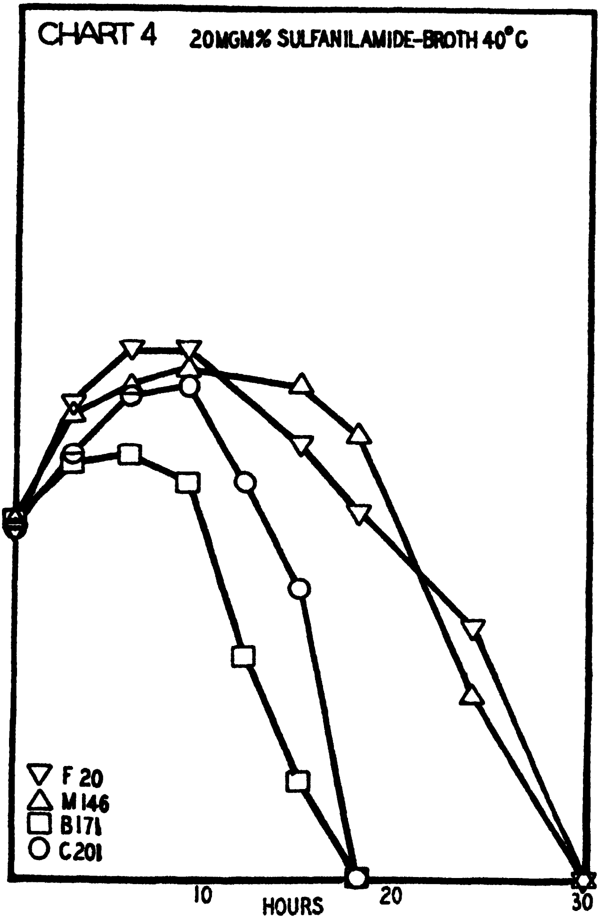
incubation being carried out at 37° and at 40°C. respectively. It is evident that at both temperatures the drug had little or no effect upon bacterial multiplication during the first three hours. This delay in the action of the drug is typical. Repeated tests with many strains have always shown a delay in the action of sulfanilamide lasting for about three hours. To date, we have been unable to determine the factors responsible for this continued multiplication of the streptococci in the presence of sulfanilamide during the initial state of incubation. Between the third and sixth hour, the beginning of an inhibitory effect upon all four strains, at both test temperatures, is apparent. This inhibitory effect has continued throughout the test period at 37°C. (Chart 3). At 40°C., de-celeration of growth rate has



continued up to the ninth hour followed by a progressive decrease in bacterial concentration to the point of sterility: in eighteen hours with the strains B-171 and C-201; in thirty hours with strains F-20 and M-146 (Chart 4).



Results obtained under our test conditions had clearly indicated that a bactericidal effect with 20 mgm. per cent sulfanilamide could be demonstrated at 40°C. We then tested a series of mixtures containing 10 mgm. per cent of the drug and obtained the results shown in table 4. It may be seen that test mixtures with an initial concentration yielding approximately 10,000 colonies per cubic centimeter were sterilized by 10 mgm. per cent sulfanilamide in 30 hours at 40°C. No attempt was made during the present study, to determine the minimal bactericidal



concentration of sulfanilamide for various sizes of initial streptococcal concentrations.

The effect of 20 mgm. per cent sulfanilamide, at 40°C., upon three different initial concentrations of each of two strains is shown in table 5. These results indicate that strain B-168 is more susceptible than B-170 to the bactericidal action of the drug. B-168 is our laboratory number for the "Richards" strain used by Colebrook and his associates (1936).

Quantitative relationships between drug and bacteria in terms of bactericidal effect at 40°C., together with the question of variation in degree of strain susceptibility will be dealt with in a subsequent report.

TABLE 4

Bactericidal effect of 10 mgm. per cent sulfanilamide upon Beta-hemolytic streptococci in PD broth at 40°C.

STRAIN	INITIAL BACTERIAL CONCENTRATION	BACTERIAL CONCENTRATION AFTER 30 HOURS	
		Sulfanilamide-broth	Broth
F-20	4.1	Sterile	7.7
M-146	4.1	Sterile	7.8
B-171	3.4	Sterile	7.0
B-171	4.1	Sterile	7.8
C-201	4.2	Sterile	7.2

Bacterial concentrations determined by plating 1.0 cc. of test mixtures in blood agar pour plates after making required dilutions in broth. Figures above indicate the logarithm_{10} of plate counts.

TABLE 5

Bactericidal effect of 20 mgm. per cent sulfanilamide upon large and small inocula of two strains of Beta-hemolytic streptococci in PD broth at 40°C.

STRAIN	INITIAL BACTERIAL CONCENTRATION PER CC.	GROWTH AFTER 48 HOURS INCUBATION	
		Sulfanilamide-broth	Broth
B-168	350,000	Sterile	++++
	35,000	Sterile	++++
	3,500	Sterile	++++
B-170	170,000	++++	++++
	17,000	Sterile	++++
	1,700	Sterile	++++

COMMENT

The mode of action of sulfanilamide, *in vitro* as well as *in vivo*, has remained obscure. Several investigators (Colebrook *et al.*, 1936; Long and Bliss, 1937 a and b; Bliss and Long, 1937; Finklestone-Sayliss, 1937) have concluded that the drug's action is directly upon the bacteria, but there has been incomplete agreement as to whether this action is truly bactericidal or merely inhibitory in nature. Our studies have been in connection with the effect of sulfanilamide *in vitro* only. Under these conditions, we believe that the effect obtained with the drug, on hemolytic streptococci, will depend principally upon the temperature at which the test is run.

The fact that bactericidal action had been observed with the drug in whole blood at 40°C., when only inhibition could be demonstrated at 37°C., led us to conclude that the temperature at which bacteria were subjected to action of the drug determined whether sterilization or inhibition would result. However, we realize that it is difficult to draw definite conclusions from results with a test mixture which contains not only drug and bacteria, but leucocytes and serum as well.

In plain PD broth, on the other hand, it seems to us that there can be no question of bactericidal action on the part of any element other than the drug itself. With such a mixture we are obviously dealing only with bacteria multiplying in an environment which includes nutritive elements, metabolites and drug. Under these conditions, it appears that temperature is the determining factor. Although we have repeatedly verified our bactericidal results at 40°C., we have, thus far, been unable to obtain data which will explain just why the streptococci are killed by the drug at 40°C. when they are merely inhibited at 37°C. under conditions otherwise similar.

The significance of a bactericidal action with sulfanilamide at 40°C. *in vitro* should not be overlooked. Bactericidal action with this drug has been demonstrated by us only at elevated temperatures corresponding to those associated with high fevers. This suggests a correlation with successful clinical treatment of patients with severe hemolytic streptococcus infections involving high temperatures. Hence, in rapidly successful sulfanilamide therapeusis, it may be that the invading bacteria have been exposed, at times, to the action of the drug at temperatures similar to those at which bactericidal action has been demonstrated *in vitro*. As far as we have been able to determine, there has been but one report on the combined use of hyperthermotherapy and sulfanilamide. Ballenger, Elder and McDonald (1937), in a clinical note have reported successful treatment of a few cases of gonococcic infection with the administration of an artificial fever of 103° to 104°F. combined with sulfanilamide. It is, perhaps, significant that patients chosen for this combined treatment were those who had failed to respond to either treatment alone.

The significance of our observations in connection with making comparative tests on sulfanilamide and related compounds, *in vitro*, is obvious.

CONCLUSIONS

Bactericidal action upon thirty-six strains of Beta-hemolytic streptococcus recently isolated from human infections has been demonstrated with 20 mgm. per cent sulfanilamide *in vitro* at 40°C. in peptone-glucose broth and in whole blood. Preliminary results indicate that this streptococcidal action can be demonstrated *in vitro* with lower concentrations of sulfanilamide.

Under our experimental conditions, bactericidal action due to sulfanilamide could not be demonstrated at test temperatures lower than 39°C.

Experimental evidence indicates that the action of sulfanilamide *in vitro* is delayed for at least three hours.

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A SYNTHETIC MEDIUM FOR THE CULTIVATION OF C. DIPHTHERIAE[1]

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Received for publication May 31, 1938

It has been shown by Pappenheimer and Johnson (1936), Pappenheimer (1936), and Johnson, Pappenheimer and Robinson (1938), that diphtheria toxin containing 50 to 60 L_f doses per cubic centimeter may regularly be produced on a medium of simple chemical composition. That this is by no means the upper limit of toxin production is demonstrated by the fact that the same strain of Park 8 produces as much as 100 L_f per cubic centimeter on a pig-stomach digest medium, Taylor (1935). It is therefore probable that the "synthetic" medium can be improved to a point where it will equal or excell the empirical one in toxin production.

The simplified medium used by Pappenheimer and co-workers is, in a general way, based on work which has been going on for some time in this laboratory concerning growth requirements of the diphtheria bacillus as a part of a general program of study of nutritive requirements of pathogenic organisms. Many of the results obtained have already been presented (Mueller and collaborators 1933–1938), and since it is now possible to complete the listing of substances essential to very heavy growth of the strain which has been most intensively studied, thus opening the way for further work on toxin production, it may not be out of place to summarize very briefly the more important points which have been established.

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[1] **Through the courtesy of various members of its Faculty, part of these experiments were carried out at the Dalhousie University Medical School, Halifax, Nova Scotia, during the summer of 1937.**

The plan of approach was to start with a known, suitable medium (meat-extract broth) and attempt to isolate or identify the chemical substances contributing to its usefulness. Fortunately, for the sake of comparison of results over a period of time, it was thought best to adopt a relatively exact method for estimating growth, and this was done by the determination of nitrogen in the centrifuged and washed bacteria from 10 cubic centimeter quantities of medium after an optimal period of incubation. Most strains of the diphtheria bacillus yield in this way from 1 to 2 milligrams of bacterial nitrogen on nutrient bouillon.

The broth was considered to be composed of two classes of compounds, (*a*) "peptones," or protein split-products of varying degree of complexity, and (*b*) muscle extractives. It was first shown that a protein such as casein, thoroughly hydrolyzed by strong acid to the amino acid stage, and in some cases reinforced by tryptophane (which is destroyed by this procedure), could be substituted for the peptone. Commercial meat extract was used as the source of extractives. Neither the meat extract alone, nor the hydrolyzed protein alone supported good growth; together, particularly when some enriching substance, e.g., maltose, ethyl alcohol, glycerol or lactic acid was added, quite satisfactory growth was obtained.

It was then possible to determine, in the case of several strains of the organism, which of the amino acids were essential, and in what quantity. Differences appeared among the various strains, although certain amino acids, such as cystine, glutamic or aspartic[2] acid, methionine and valine were required by all or most strains.

The meat extract fraction was next examined. It was found, as might have been anticipated, that certain essential inorganic materials were supplied by it, i.e., Mg, K, and PO_4. In addition, however, organic substances were involved which were effective in extremely small amounts. Various liver extract fractions were actually used at this stage, by-products from preparations of the pernicious anemia curative material, obtained through the courtesy and cooperation of Dr. Y. Subbarow of the Department of Biological Chemistry, Harvard University Medical School and of the Lederle Laboratories, Inc.

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[2] Aspartic acid appeared not to be used by the first strain which was reported ("HY"). Later work indicated that some strains prefer glutamic acid, others aspartic acid, and others do best on a mixture of the two.

With this material it was shown that the essential organic materials could be separated into two fractions by ether extraction from acid solution. The ether-soluble material, found also to occur in high concentrations in the urine of herbivors, was isolated from cow's urine and identified as pimelic acid, a substance which had previously not been known to have biological importance. The material not extracted by ether was shown to contain two essential compounds. These were obtained from liver extract by esterification and acetylation, and in this form, careful vacuum distillation effected the separation. One was isolated in pure crystalline condition and identified as nicotinic acid, which had just been shown by Knight (1937) to be essential to the growth of the staphylococcus. The other, although not actually isolated, had properties which led to the belief that it might be β-alanine, which had previously been shown by Williams and Rohrman (1936) and by Miller (1936) to be essential to yeast growth. This proved to be the case, and the three substances pimelic acid, nicotinic acid and β-alanine, together with suitable amino acids, inorganic salts, and sodium lactate, yielded growth of approximately 2.25 mgm. bacterial nitrogen per 10 cubic centimeter of medium. It is on substrates of this type that Pappenheimer and co-workers have been able to produce potent toxin with a strain of the Park 8 organism when care was taken to remove excess iron, which occurs in all the materials used as an impurity.

While such growth was good, it was not as heavy as had been obtained with more impure materials; 3.5 mgm. N had frequently been observed, and both from the theoretical side of the matter, and the practical one of perhaps increasing the toxin yield, the work has been continued to learn the conditions under which better growth can be obtained.

It may at this point be again emphasized that there are many differences in nutritive requirements between strains of *corynebacterium diphtheriae.* Even between strains of supposed Park-Williams no. 8 obtained from various laboratories, these differences are striking, and involve amino acids, sources of energy, e.g., lactic acid, glycerol, etc., and the accessory substances. Thus, while β-alanine and nicotinic acid seem to be required by practically all strains, pimelic acid increases the growth of only about half the strains so far tried and is without effect on the others. Moreover, two strains, both Park 8, one from Albany, N. Y., the other from Utrecht, Holland, have been encountered, which fail to grow on such media, and evidently have some further or different requirements. Whether all these differences within what is supposed to be a single strain represent variation over a period of many years, or whether other organisms have in some instances been substituted for the Park 8, there is at present no means of knowing. It is important that it be realized, however, that the medium to be described in this paper will not produce similar results with all strains of the organism.

The work which is to be described has been carried out on our "Allen" strain (not a Park 8), which has been used and described in an earlier paper. It gives a positive virulence test in guinea pigs, but produces practically no toxin, under conditions favorable to toxin production with the Park 8 used at the Massachusetts State Antitoxin Laboratory. It has the great advantage of growing rather more rapidly than the Park 8, making possible an earlier assessment of experimental results.

It is now possible to obtain approximately 9.0 mgm. bacterial nitrogen with this strain from 10 cc. of medium. The increase from the 2.25 level originally obtained, to the present one, has been, the result of increasing the quantity of two of the materials used previously,—*cystine* and *lactic acid,* and the addition of traces of suitable *heavy metals,*—iron, manganese, copper and zinc. The experiments may be discussed under these three headings but since the final result depends upon the proper adjustment of all three, it is naturally impracticable to describe experiments under each heading involving only that one material or group. In the interest of brevity, therefore, only a few crucial experiments will be given in detail, which will illustrate the effect of all the substances concerned.

CYSTINE

Although 1.0 mgm. of cystine in 10 cc. of medium was more than sufficient for maximal growth when meat or liver extract was used in the medium, with purified extractive substances it became, at that concentration, the limiting factor. With all other components present in optimal amount, experiments in which increasing quantities of cystine are used show maximum growth to require from 6.0 to 8.0 mgm. of the amino acid in 10 cc. At a concentration of 8.0 mgm. or more, there is considerable danger of separation of the cystine in crystalline form. Up to that point the other amino acids are quite able to hold it in solution, in spite of its low solubility in pure water. One merely adds it as the hydrochloride to the other components of the medium, adjusting the reaction to pH 7.6 with NaOH only when everything else is added and the mixture has been diluted nearly to its final volume. If it is permitted to crystallize out, it will not redissolve during autoclaving, and the medium cannot be used.

It is possible that this increased requirement for cystine represents an additional function of the cystine in replacing some factor present in the tissue extract, although no direct evidence on the point has been obtained. There is no doubt that a portion of its action is due to an effect on the oxidation-reduction potential of the medium, but this is probably not the full explanation. In view of the simultaneous requirement for methionine shown by all strains so far examined, the sulfur metabolism of the organism must be complex.

LACTIC ACID

The substance "lactic acid" as commonly purchased, is a syrupy solution, marked as containing 85 per cent or somewhat more of the pure chemical. If this commercial material be added to a culture medium, the pH adjusted and the medium autoclaved, it will invariably be found to have turned acid. In most of this work, a sodium lactate solution has been prepared by diluting the commercial acid with about an equal volume of water, adding phenol red, and neutralizing with 35 to 40 per cent NaOH to distinct alkalinity. The very hot solution is then boiled for a few minutes, adding more NaOH drop by drop when the purple color begins to turn red, until the color is permanent. The solution is then diluted to four times the original volume of 85 per cent lactic acid. This solution keeps indefinitely and is added to media as required. The explanation for the failure of the acid to be sharply neutralized probably lies in the fact that the commercial material contains a considerable amount of lactic anhydride, which is rather slowly converted to the acid on dilution and neutralization. Recently, however, owing to anomalous results in growth with two new lots of lactic acid, it seemed necessary to inquire more carefully into the nature of the material marketed as "lactic acid."

The commercial product is prepared by fermentation. Certain organisms are known to produce mostly *d*-lactic acid, the form which occurs in muscle, and known also as "sarcolactic" acid. Other organisms form the laevo isomer. One would expect, therefore, in commercial preparations that both *d*- and *l*-lactic acid would occur, but not necessarily in equivalent proportions, unless the material were completely racemized at some step in the refinement.

Two different bottles of commercial lactic acid were therefore examined for optical rotation, and each found to have a slight effect on polarized light. In order to insure the absence of metals, ethyl lactate was prepared from each of these and also from a third specimen secured in the meantime. The three ethyl lactates boiling at about 70° under 36 mm. pressure gave the following optical rotations, in a 1 dm. tube: I, −6.22°; II, +1.93°; III, −6.47°. Beilstein gives for *d*-lactic $[\alpha]_D^{14}$ - 10.33°. Other sources give slightly larger figures up to 13° or even 14°. It is to be noted that the direction of the rotation of the acid is reversed with the ester.

A specimen of d-calcium lactate from the U. S. Department of Agriculture yielded a preparation of ethyl lactate showing a rotation of -8.62°, indicating that some racemization took place during the preparation.

Taking the figure −10.33 as 100 per cent optical activity for the substance, and without making the slight corrections for temperature and density, it may be calculated that the various specimens of lactic acid had approximately the following composition: I, 80 per cent *d* and 20 per cent *l*; II, 41 per cent *d* and 59 per cent *l*; III, 81 per cent *d* and 19 per cent *l*; U. S. Dept Agr., 92 per cent *d* and 8 per cent *l*.

The ethyl lactates were converted to sodium lactate by adding water and NaOH, with warming until a permanent alkaline reaction was reached, and were then subjected to vacuum distillation to remove the alcohol.

Using a casein hydrolysate medium like formula *A*; (see final section) but containing lactates from the above three different commercial specimens, and also one from pure *d*-lactic acid, the following results were obtained: (1) Control with lactate I, equivalent to 0.15 gram lactic acid, 7.02 mgm. N. (2) Control with lactate II, equivalent to 0.15 gram lactic acid, 5.57 mgm. (3) Control with lactate III, equivalent to 0.15 gram lactic acid, 7.38 mgm. (4) Control with pure *d*-lactic acid, 0.15 gram, 8.10 mgm.

Similar results were obtainable with great regularity, the amount of growth always quite parallel with the proportion of the dextro acid present.

A further experiment, designed to test directly the utilization of *l*-lactic acid, was then carried out. This material is not commercially available, and if it is not utilized by the diphtheria bacillus, it seemed probable that it could be isolated from cultures grown on commercial lactic acid from which the *d* form had been used up. For this purpose, sample II (above) of lactic acid, containing a slight excess of *l* over *d* was used.

Two liters of the casein hydrolysate medium (formula A) were prepared, containing 1.75 per cent of this commercial acid, sterilized in several Kolle flasks and inoculated with the Allen strain. After four days' incubation, flasks and contents were steamed, and the bacterial growth centrifuged out. The supernatants were concentrated *in vacuo* to about $\frac{1}{5}$ the volume, strongly acidified with H_2SO_4 and extracted with butyl alcohol. The alcohol was then extracted with NaOH solution and again used for a second extraction of the medium. In all, six double extractions were carried out, and then the combined NaOH extracts, containing the lactic acid as well as other materials, were concentrated in vacuum, acidified with H_2SO_4 and extracted continuously with ether. Evaporation of the ether yielded a strongly acid syrup, from which some crystalline material, identified as succinic acid, separated. The remainder was converted to the zinc salt and fractionally crystallized. After removal of an initial crop of zinc *dl* lactate, an abundant crop of material was obtained, which after two recrystallizations was found to be pure zinc *l* lactate (correct optical rotation and water of crystallization.)

This material, freed from zinc as described below, was tested for its effect on growth of the test organism, using again a medium of formula *A*, but containing no lactic acid. For comparison, a similar set to which *d*-lactic acid was added is also given. In each case, of course the compound is added as the Na salt.

	gram	*mgm. N*
1. Control +		2.29
2. Control + *l*-lactic acid	0.025	1.50
3. Control + *l*-lactic acid	0.05	2.60
4. Control + *l*-lactic acid	0.1	3.06
5. Control + *l*-lactic acid	0.125	3.12
6. Control + *l*-lactic acid	0.15	2.82
7. Control + *l*-lactic acid	0.175	2.60
8. Control + *d*-lactic acid	0.025	4.49
9. Control + *d*-lactic acid	0.05	5.70
10. Control + *d*-lactic acid	0.1	7.76
11. Control + *d*-lactic acid	0.125	8.80
12. Control + *d*-lactic acid	0.15	8.26
13. Control + *d*-lactic acid	0.175	9.12

In spite of the irregularity in tubes 2, 11 and 12 the results are perfectly clear-cut. The moderate increase shown with *l*-lactic acid may be due either to slight racemization during autoclaving, or to a direct effect on the part of the organism.

It is clear from all the evidence that, in the case of this strain, utilization of lactic acid is pretty definitely limited to the *d* form occurring naturally in body tissues. Whether this will hold for other strains of *C. diphtheriae* remains to be determined. The amount of growth appears to increase up to the point at which the concentration becomes inhibitory, perhaps through osmotic effects or an excess of Na. This concentration (the equivalent of approximately 2.0 per cent lactic acid) seems to be independent of the optical form of the material and consequently, the greater the proportion present in the *l* form, the less is available for growth. The use of a fairly pure *d*-lactic acid, therefore, leads to much higher levels of growth than is possible with the commercial acids.[3]

HEAVY METALS

At a very early stage of these studies it was recognized that significant amounts of phosphorus were furnished to the medium by meat extract. Later, with the use of charcoal-adsorbed material, potassium and magnesium deficiencies appeared, and were overcome by changing the formula of the salt mixture to contain quantities within the optimal range of concentration. Iron salts, included in the earlier media, had been omitted when the work of Pappenheimer and Johnson showed this element already to be present in the reagents in sufficient concentration to inhibit toxin formation.

The substitution of β-alanine, nicotinic and pimelic acid in minute quantities for tissue extract of course withdrew a very important source of mineral impurity and made necessary a careful investigation of the effects, not only of iron, but of other elements. This has been carried out simultaneously with the experiments described above, and has proved to be exceedingly troublesome.

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[3] The zinc salt of *d*-lactic acid, a generous supply of which was obtained from the Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, through the courtesy of Dr. G. E. Ward, may be dissolved in hot water to form an approximately 10 per cent solution, the zinc removed with H_2S, the precipitate washed thoroughly with hot water, and the lactic acid extracted with ether after moderate concentration in vacuum. The ether extraction is only necessary in order to insure minimum contamination with zinc, etc.

It was possible at any stage to show the necessity for Fe, when the Wadsworth and Wheeler (1934) technic of precipitating calcium phosphate in the medium, (shown by Pappenheimer and Johnson to remove excess Fe) was carried out. Iron salts alone were usually incapable of re-activating such treated media to the point where as good growth could be obtained as was given before the calcium treatment. The addition of hay ash, or the ash of other organic materials, in suitable concentration, almost always improved the growth when optimal, but not excessive, quantities of Fe were added, (as $FeCl_3 \cdot 6H_2O$ or $FeSO_4 \cdot 7H_2O$).

Numerous separations of the constituents of such ash materials were carried out by the usual methods of qualitative inorganic analysis, selecting procedures which were applicable in the presence of Ca and PO_4. The various fractions so obtained were tested for their effect on growth. Clear-cut results were difficult to obtain, a fact which was explicable in several ways: (*a*) Traces of many other elements than Fe undoubtedly occurred in the other components of the medium. (*b*) While the calcium treatment could be shown to remove most of the iron, it did not follow that the same would be true for other substances, or even that quantities of the same order of magnitude were present or required. (*c*) It was uncertain how effectively the analytical methods separated the usual metallic ions in the presence of relatively large amounts of Mg, Ca, PO_4, K and Na. (*d*) The extent to which rarer elements might be involved could not be foreseen.

As an illustration of these difficulties it may be noted that spectroscopic examination of one of the ash materials examined, (prepared from the butyl alcohol residue of a fibrin hydrolysate, kindly supplied by Prof. W. C. Rose of the Department of Biological Chemistry, University of Illinois) showed the presence of the following elements:

Ca
Al
Fe
Cu
Mg
Si
Mn
K
Na
Pb
B
In
Sn
Sb
Ba
Ag
Li
P
Sr
Mo
Ni
Zn
Ga
Cr

(Analysis done by Mr. Rockwell Kent, III, of the Massachusetts Institute of Technology.)

No effort has been made to purify the various organic and inorganic materials used in the preparation of test media. It was felt that any attempt to remove all the possible traces of foreign elements to a point two or three orders of magnitude below the range in which Fe, for example, inhibits toxin formation would be foredoomed to failure. That glassware, itself, may supply significant amounts of inorganic material is clearly indicated in the work of Pappenheimer and Johnson already cited. It seemed wiser to work the matter out as thoroughly as possible with materials readily and generally obtainable, leaving further refinements for future work, should it be indicated.

The results of numerous experiments with various types of control indicate quite clearly that the elements iron, manganese, copper and zinc are invariably essential for maximal growth, and further increases have been caused frequently, but not reproducibly, by cobalt, nickel and arsenic. Iodine and boron, probably also essential to protoplasmic development in general, have no effect on the growth of the test strain of diphtheria, and both undoubtedly occur as impurities in the medium in adequate concentration. In view of the striking effect of Fe on toxin production, as well as on growth, the whole subject of mineral requirements must naturally be re-investigated in relation to both matters with a standard toxin-producing organism. It seems unwarranted to extend the experiments with the Allen strain, and it is proposed to discontinue the work with it at this point.

It is not without interest or perhaps significance that these same metals, Fe, Mn, Cu and Zn (also Co) are now recognized as essential to many types of plant growth (see Biochemical Reviews 1935 and 1936) and that, in addition, the plant physiologists, faced with the same difficulties of the occurrence as impurities of minute amounts of many elements, make use of the so-called "A-Z" solutions which contain traces of twenty or more additional elements, and in that way frequently obtain better growth.

As a result of many experiments, the following quantities of the various metals appear to be suitable.

	γ per cc.
$FeSO_4 \cdot 7H_2O$	5.0 (would be inhibitory to toxin formation
$MnSO_4 \cdot 4H_2O$	2.5
$CuSO_4 \cdot 5H_2O$	5.0
ZnO (dissolved in HCl)	2.5

Strikingly clear-cut experiments showing the effect of these various elements are not always obtained. The treatment with calcium evidently removes Fe quite effectively so that the concentration of this element giving optimal growth can be determined with some accuracy. The same is not true of the other metals, and the amounts employed may be considerably in excess of the actual requirements. They are, however, well below concentrations inhibitory to growth.

The following experiment illustrates the effect of Fe:

The basic medium was made according to formula A (last section), omitting the Fe. In order to remove the Fe present as impurities, all the constituents of the medium except the $MgSO_4$, which would be precipitated, the pimelic and nicotinic acids and β-alanine, which might be adsorbed on the precipitate, and the ethyl alcohol (volatile), were mixed, diluted to about ⅔ the final volume and brought to about pH 8.4. For each 10 cc. lot, 3 mgm. $CaCO_3$ (dissolved in HCl) were added, and the solution boiled a few minutes and filtered through ashless paper. To the cooled filtrate the same amount of $CaCl_2$ was added and the procedure repeated. After adding the $MgSO_4$, accessories and alcohol, the filtrate was diluted to the desired volume, distributed in tubes and autoclaved at 10 pounds for 10 minutes. When cool, the Ca, Mn, Cu and Zn salts were added sterilely, and, finally, sterile $FeSO_4 \cdot 7H_2O$ as indicated in the table.

The tubes were incubated at 34° for 80 hours. Nitrogen determinations on the washed bacterial growth are given at the right.

	γ	*mgm. N*
1. Control without $FeSO_4 \cdot 7H_2O$		0.58
2. Control plus $FeSO_4 \cdot 7H_2O$	0.5	1.22
3. Control plus $FeSO_4 \cdot 7H_2O$	1.0	3.44
4. Control plus $FeSO_4 \cdot 7H_2O$	2.5	4.01
5. Control plus $FeSO_4 \cdot 7H_2O$	5.0	6.09
6. Control plus $FeSO_4 \cdot 7H_2O$	10.0	8.26
7. Control plus $FeSO_4 \cdot 7H_2O$	25.0	9.23
8. Control plus $FeSO_4 \cdot 7H_2O$	50.0	9.75
9. Control plus $FeSO_4 \cdot 7H_2O$	100.0	9.60

It appears that the medium still contained the equivalent of approximately 0.25 γ $FeSO_4 \cdot 7H_2O$ per 10 cc. lot. The two calcium phosphate precipitates (from a total of 30 lots) were shown to contain approximately 0.54 mgm. Fe in the first, and 0.02 mgm. Fe in the second. This is equivalent to a $FeSO_4 \cdot 7H_2O$ concentration of nearly 100 γ per lot originally present as impurities.

The iron could not always be removed as completely as was the case in this experiment and individual experiments with the other metals were often irregular and unsatisfactory. The entire matter may therefore be summed up in a further experiment which shows the effect of omission from the completed media of each in turn of the metals under consideration. The compositions of formulae *A* and *B* are given in the final section of the paper, and the general procedure was identical with that described above, including two precipitations with calcium phosphate.

	mgm. N
1. Medium A complete	8.15
2. Medium A without Fe	3.82
3. Medium A without Mn	7.88
4. Medium A without Cu	5.40
5. Medium A without Zn	6.01
6. Medium B complete	7.04
7. Medium B without Fe	2.76
8. Medium B without Mn	6.66
9. Medium B without Cu	4.31
10. Medium B without Zn	4.35

GENERAL SUMMARY

In order to bring together all the facts discussed above, as well as to summarize and compare the part played by each component of the medium, two further experiments may be given. In the first, a casein hydrolysate medium (*A*) was used, while pure amino acids replaced this material in the second (*B*). The composition of the two complete media may be seen from the protocols themselves. The first tube in each experiment is complete, while from each successive tube, a single material is omitted; all others being present in the quantities shown, in a volume of 10 cc. The $CaCl_2$ treatment was not used in this case, since it would have been too cumbersome, and the results indicate the presence of Fe, Mn and Zn present as impurities.

Formula A

	gram	*mgm. N*
1. Complete medium		8.90
2. Complete medium without casein hydrolysate	0.1	0.49
3. Complete medium without aspartic acid	0.025	8.32
4. Complete medium without glutamic acid hydrochloride	0.025	8.50
5. Complete medium without cystine	0.006	0.84
6. Complete medium without tryptophane	0.0001	8.92
7. Complete medium without KCl	0.004	5.22
8. Complete medium without Na_2HPO_4	0.3	4.62
9. Complete medium without $MgSO_4 \cdot 7H_2O$	0.01	1.81
10. Complete medium without pimelic acid	0.000,001,5	5.30
11. Complete medium without nicotinic acid	0.000,023	0.20
12. Complete medium without β-alanine	0.000,023	0.21
13. Complete medium without ethyl alcohol	0.07 (cc.)	6.85
14. Complete medium without *d*-lactic acid	0.175	2.44
15. Complete medium without $CaCO_3$ (in HCl)	0.002	7.92
16. Complete medium without $FeSO_4 \cdot 7H_2O$	0.000,05	8.21
17. Complete medium without $MnCl_2 \cdot 4H_2O$	0.000,025	8.00
18. Complete medium without $CuSO_4 \cdot 5H_2O$	0.000,05	5.80
19. Complete medium without ZnO (in HCl)	0.000,025	9.12

In addition, an amount of NaCl varying between perhaps 0.2 and 0.4 gram per lot is present, resulting from the neutralization of HCl in the various solutions by NaOH.

Formula B

	gram	*mgm. N*
1. Complete medium		7.68
2. Complete medium without *l*-cystine	0.007	0.26
3. Complete medium without *dl*-valine	0.02	6.90

		gram	mgm. N
4.	Complete medium without *l*-methionine	0.006	0.67
5.	Complete medium without *l*-tyrosine	0.005	7.84
6.	Complete medium without *l*-proline	0.0075	7.04
7.	Complete medium without *l*-aspartic acid	0.05	4.27
8.	Complete medium without *d*-glutamic acid hydrochloride	0.075	4.94
9.	Complete medium without KCl	0.004	1.97
10.	Complete medium without Na_2HPO_4	0.3	0.06
11.	Complete medium without $MgSO_4 \cdot 7H_2O$	0.01	0.26
12.	Complete medium without pimelic acid	0.000,001,5	6.22
13.	Complete medium without nicotinic acid	0.000,023	0.10
14.	Complete medium without *β*-alanine	0.000,023	0.13
15.	Complete medium without ethyl alcohol	0.07 (cc.)	6.34
16.	Complete medium without *d*-lactic acid	0.175	0.39
17.	Complete medium without $CaCO_3$ (in HCl)	0.002	4.14
18.	Complete medium without $FeSO_4 \cdot 7H_2O$	0.000,05	7.24
19.	Complete medium without $MnCl_2 \cdot 4H_2O$	0.000,025	7.49
20.	Complete medium without $CuSO_4 \cdot 5H_2O$	0.000,05	5.73
21.	Complete medium without ZnO (in HCl)	0.000,025	7.40

It may be readily observed to what extent the complete media are dependent upon each of their individual components. The presence of casein hydrolysate in formula *A* naturally obscures the picture in the case of certain of the items, such as cystine, aspartic and glutamic acids, K, PO_4, Mg, and Ca, because of the presence of larger or smaller quantities as impurities in the commercial casein.

The amino acid formula (*B*) does not produce quite as heavy growth as does the casein hydrolysate formula. It has not seemed worth while to spend more time in attempts to improve it, because of the variation in amino acid requirements between strains and the fact that the Allen strain is not to be further investigated at this time. It is evident that neither valine, tyrosine nor proline, individually, exert any very marked effect. In numerous other experiments the general trend of results has indicated a distinct advantage when all are present. Moreover, the addition of several additional amino acids, together, usually gives somewhat better growth. Probably at this level the less that is demanded in the way of gross synthetic processes the better the growth that will result. To what extent these results can be applied to the production of toxin, and wherein lie the differences in toxin-producing ability of different strains of the diphtheria bacillus, would seem to be problems which can now be approached with the aid of reproducible media.

CONCLUSION

Growth of a strain of diphtheria bacillus, previously giving approximately 2.25 bacterial nitrogen on 10 cc. of a medium of simple and largely known composition can be increased three to four fold by increasing the proportions of certain of the components, notably cystine and *d*-lactic acid, and by adding traces of Fe, Mn, Cu and Zn.

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FLAGELLA STAINING AS A ROUTINE TEST FOR BACTERIA[1]

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Received for publication May 31, 1938

Nearly all descriptions of bacterial species contain statements as to whether or not motility is observed. The unsatisfactory character of this test has been realized for some time and various efforts have been made to improve upon the usual method, i.e., the microscopic examination of a hanging-drop. The most notable attempt of this sort in recent years is that by Tittsler and Sandholzer (1936), which is a macroscopic test depending upon the type of growth produced in a semi-solid agar.

In employing this semi-solid agar test on a series of soil organisms, certain plant pathogens, and organisms of the *Alcaligenes fecalis* and violet chromogenic groups, it was soon observed that, just as claimed by its originators, the test picks out many organisms that would not be recognized as motile by examination under the microscope. It has two disadvantages, however: it is rather slow, as cultures must be kept 3 or 4 days before a satisfactory reading can be made; it is uncertain, for considerable experience is necessary before it can be properly interpreted, and even an experienced user of the technic finds many tubes which give indefinite results. There proved, moreover, to be numerous cultures which appeared immotile in hanging-drop, and gave somewhat indefinite results by the semi-solid agar test, but which were shown on staining to have unmistakable flagella.

This raised the question as to whether it might be possible to devise a flagella-staining technic simple enough to be employed in routine pure culture study in the place of the conventional test for motility. This has never been tried in the past because flagella staining methods are notoriously complicated and often fail to give identical results in the hands of different workers. Very recently, however, Hofer and Wilson (1938) have proposed a modification of the Gray flagella stain especially adapted for application to slime-forming bacteria. The results obtained with this technic were so consistent and were obtained with so little trouble, even in the hands of inexperienced technicians, that it seemed hopeful to modify the technic slightly so as to apply to other than slime-forming organisms. It was found easily possible to do this, the principal modification being the length of time to allow the organism to stand in water before making the smear on the slide. The procedure now employed in the case of miscellaneous bacteria is as follows:

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[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 270.

PREPARATION OF GLASS SLIDES

Use new slides if possible. (This is because under the drastic method of cleaning to remove grease, old slides have a greater tendency to break, due to crystallization of the glass.) Clean first in a dichromate cleaning fluid, wash in water and rinse in 95 per cent alcohol; then wipe with a clean piece of cheese cloth. (Wiping is not always necessary but is advisable unless fresh alcohol is used after every few slides.) Pass each slide back and forth through a flame for some time, ordinarily until the appearance of an orange color in the flame; some experience is necessary before the proper amount of heating can be accurately judged.

Cool slides gradually in order to re-anneal, and thus to minimize breakage. A crude but ordinarily satisfactory method of annealing is to place the flamed slides on a metal plate (flamed side up) standing on a vessel of boiling water; and then to remove the flame under the water so as to allow gradual cooling. (Failure to anneal may result in breakage, sometimes as long as two weeks after the heating.)

PREPARATION OF SUSPENSIONS

Use young and actively growing cultures (e.g., 18–22 hours old), on agar slants. With a flamed *but well-cooled* loop transfer a small amount of growth to 5-10 cc. of sterile distilled water, which has been held for several hours at room temperature. (Poor slides result from suspensions made in water that is too hot or too cold.)

Mix thoroughly in the distilled water and allow to stand 5-30 minutes, according to the type of growth produced. Gum-forming bacteria require 30 minutes, as recommended by Hofer and Wilson; those that produce no gum must stand in the water only 5-10 minutes. Standing in the water should be just long enough to allow the flagella to become untangled; too long a time results in their breaking off.

With a loop that has been flamed *and cooled*, remove a drop from the top of the suspension and place it on a glass slide prepared as above described. (The reason for taking material from near the surface of the suspension is because the non-motile cells tend to settle, while the motile cells– at least in the case of aerobes– collect at the top.) Smear the drop over the slide with the use of a second slide, as in preparing blood films; the film should be thin enough to dry rapidly and thus to minimize distortion.

STAINING

Use the mordant recommended by Gray (1926): 5 cc. saturated aqueous solution potassium alum; 2 cc. saturated aqueous solution mercuric chloride; 2 cc. 20 per cent aqueous solution tannic acid; 0.4 cc. saturated alcoholic solution basic fuchsin (presumably about 6 per cent, which was the strength employed in the present work). For best results, filter just before using. The technic is essentially that of Gray. Apply cold for 8-10 minutes; 10 minutes is ordinarily best, but this varies with the organism studied. (More than 10 minutes of mordanting is apt to cause too much precipitate.)

After mordanting, wash slides about 10 seconds in running water. Dry in the air, without heating. Stain 5 minutes, without heating, with Ziehl's carbol fuchsin; wash in running water; dry and examine. (In the present work a recently certified batch of National Aniline basic fuchsin was employed.)

In this procedure the most crucial points are the proper amount of heat to give the slides and the method of cooling so as to minimize the tendency to break. Although not all breakage can be avoided, the above-described method of cleaning the slides is recommended because of the better demonstration of flagella than when the preparations are made on slides cleaned by more ordinary procedures.

The technic thus developed proves simple enough so that it seems possible to recommend it as a routine test. It takes longer, to be sure, than the conventional hanging-drop test; but it gives a much higher percentage of positives. Results can be obtained much more quickly than by the semi-solid agar test of Tittsler and Sandholzer; they are more reliable, because interpretation of the results is never open to question; and finally, information is thus secured not only in regard to motility but also as to the type of flagellation. It is, in fact, hardly more difficult than the Gram stain, giving results that are more clear-cut and more easily interpreted.

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THE PROTEOLYTIC ENZYMES OF BACTERIA

II. THE PEPTIDASES OF SOME COMMON BACTERIA[1]

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Received for publication May 31, 1938

INTRODUCTION

A great deal of work has been done on the proteolytic enzymes of bacteria, especially those enzymes which are secreted into the medium by the living cell or as the result of its autolysis. The proteolytic enzymes of the cells themselves, however, have been less studied. Studies on bacterial peptidases in which substrates of known formulae (such as synthetic peptides) have been used, are few and fragmentary. In most cases, only a few data illustrating the fact that the peptides were hydrolyzed are given. A brief literature review is given in table 1.

In view of this lack of information, it was decided to investigate how the peptidase systems of bacteria of widely differing genera compared with one another and with the systems found in molds (Johnson and Peterson, 1935; Berger, Johnson and Peterson, 1937), yeast (Grassmann, 1930; Macrae 1933), and animal erepsin (Johnson, Johnson and Peterson, 1936; Johnson 1937). The first paper of this series (Berger, Johnson and Peterson, 1938) described in some detail the properties of the peptidases of *Leuconostoc mesenteroides*. The present paper will give a survey of the peptidases found in twelve organisms which were chosen so as to represent aerobic, anaerobic, facultative,

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[1] Supported in part by a grant from the Wisconsin Alumni Research Foundation.

Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

proteolytic and non-proteolytic bacteria. The organisms were obtained from the stock cultures of the Bacteriology Department[2] and were as follows: *Escherichia coli*, *Bacillus megatherium*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus mesentericus*, *Bacillus subtilis*, *Clostridium butylicum* 21, *Clostridium sporogenes*,

TABLE 1

Literature review of bacterial peptidases

ORGANISM	SUBSTRATE HYDROLYZED	pH OPTIMUM	AUTHOR
Staph. pyogenes-aureus	Glycyl-*l*-tyrosine	(7)*	Otsuka, 1916
B. prodigiosus	Glycyl-*l*-tryptophane		
B. pyocyaneus†	Leucylglycine	8.4	Gorbach, 1930
	Leucyldiglycine	8.4	
B. casei	Leucylglycine	(7.5)	Tarnanen, 1930
	Glycylalanine	(7.5)	
Enterococcus, Gorini	Leucylglycine	(7.8)	Gorini, Grassmann, Schleich, 1932
	Leucyldiglycine	(7.0)	
Mammococcus, Gorini	Leucylglycine	4.8	Gorbach and Ulm, 1935
B. fluorescens	Diglycine	8.4	Gorbach and Pirch, 1936
	Leucylglycine	8.4	
	Leucyldiglycine	8.4	
Cl. histolyticum†	Leucyldiglycine	(7.8)	Weil and Kocholaty, 1937
Caseicoccus, Gorini	Leucylglycine	4.8, 7.0	Gorbach, 1937
Gastrococcus, Gorini	Leucyldiglycine	4.8	
Many aerobes, anaerobes†	Leucylglycine	(7.8)	Maschmann, (6 papers 1937–38)
	Leucyldiglycine	(7.2)	
Staph. aureus	Benzoyldiglycine	8.0	Imaizumi 1938a, 1938b
Staph. aureus, B. typhi	Leucyldiglycine	8.0	
Staph. aureus, B. typhi and *B. subtilis*	Chloroacetylphenylalanine	7.0	
14 organisms	Glycylphenylalanine	(8.0)	
14 organisms	Diglycine	8.0	
6 organisms	Benzoyldiglycine	(8.0)	

* Figures in parentheses represent the pH values at which the determination was made, a pH optimum not having been determined.

† Culture filtrates were used as enzyme source; in case of other organisms cell extracts were used.

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[2] The authors with to thank Dr. Elizabeth McCoy and Miss Elizabeth Krauskopf for supplying transfers of these organisms.

Clostridium acetobutylicum (Pike strain), *Lactobacillus pentosus* 124-2, *Propionibacterium pentosaceum* P-11 and *Phytomonas tumefaciens.*

EXPERIMENTAL

Methods

When this survey was undertaken, it was recognized that if any one procedure were adopted, it could not represent the optimum conditions for the study of all the organisms. The extent to which different organisms would vary in the nature of their peptidases was, of course, unknown, but, as a first approximation, it was assumed that their properties would fall between the limits of those of the peptidases of molds, yeast and erepsin. For example, hydrolyses were carried out at pH 8 since known peptidase systems act optimally close to this value. Since a certain degree of uniformity was desirable, it was decided to keep constant as far as practicable, the composition of the medium, the growth period of the organisms and the method of enzyme extraction. Because of these limitations, the data reported in this paper are applicable only for the conditions and methods by which they were obtained.

The organisms were grown in 20-liter Pyrex bottles, each containing 14 liters of 0.5 per cent glucose, 0.5 per cent Bacto-peptone medium made with tap water. The pH of this medium was 6.8. Six hundred cubic centimeters of a 24-hour culture grown in the same medium were used as inoculum. All cultures were incubated at 37° for 24 hours; the cells were collected by centrifuging with a Sharples supercentrifuge and then placed in the freezing compartment of a refrigerator. These wet cells usually contained 80 to 85 per cent water. In the case of the anaerobes, no special precautions were taken to preserve anaerobiosis since the depth of the medium in the Pyrex bottles was about 30 cm. In the case of the aerobes (the first six organisms listed above), the medium was aerated throughout the whole period of incubation by bubbling through it filtered, sterile air at a rate which was limited by the amount of frothing. Unfortunately, this varied with the different organisms. In all cases the cells were examined microscopically for freedom from contamination before harvesting.

Extraction of cells. With *Bacillus megatherium*, a number of extraction methods were studied. The results of these experiments can be stated briefly as follows: Peptidases were extracted from fresh cells with difficulty (in keeping with the experience of many other investigators), but repeated freezing and thawing of the cells made the enzymes readily available for extraction by a number of procedures. These included autolysis in 40 per cent aqueous glycerol, autolysis under toluene of cells previously dried by acetone-ether treatment, and autolysis of wet cells with ethyl acetate and ether. Preliminary studies on the extraction of peptidases from *Escherichia coli* gave results very similar to those obtained above. On the basis of experiments with *Bacillus megatherium* and *Leuconostoc mesenteriodes* it was concluded that keeping bacteria in a frozen condition as long as three months did not result in a decrease in peptidase activity. It was therefore decided to use the following method in general for all the organisms:

Cells were frozen and thawed at least 5 times over a period of not less than a week, and then ground with sand in a mortar. Toluene was added and the pH was maintained at 6.8 to 7.0 by the careful addition of 0.1 N NaOH. The mass was then diluted to a volume which was roughly 5 to 10 times that of the cells and the suspension was allowed to extract at room temperature, usually for 24 hours. The solution was centrifuged in an angle head centrifuge at 4000 R.P.M. until it was clear. This crude enzyme solution was then analyzed for its ability to hydrolyze various substrates. In some cases, where the cells were poor in peptidases or the efficiency of the extraction was low, it was necessary to concentrate the crude enzyme solution *in vacuo* below 40°, in order to obtain a solution of sufficient activity to make determinations within reasonable lengths of time. This concentrate was then dialyzed against distilled water, usually for about 15 hours, to remove some of the impurities. The enzyme solutions were stored in a refrigerator at 2° to 5° in the presence of toluene. Whenever an enzyme-substrate mixture was incubated for a long period where bacterial growth might occur, toluene was always added.

The extent of hydrolysis of the peptide substrates used was determined by the Linderstrøm-Lang titration (Linderstrøm-Lang, 1927). In all cases the substrate concentration was M/30 (M/15 for racemic peptides). Unless otherwise mentioned, the substrates were used at pH 7.8 to 8, being half neutralized with NaOH. They contained no added buffers. For all determinations of pH optima for hydrolysis of peptides, the regular substrates were adjusted to the desired pH by the addition of predetermined amounts of 1 N acetic acid or 1 N NaOH solution. Thus, there was no buffering other than that provided by the peptides themselves in the neighborhood of pH 8 and by the small amount of acetate at the lower pH values. The pH values of the enzyme-substrate mixtures were determined after the incubation period by means of the glass electrode. One cubic centimeter samples were titrated from 3.0 cc. of reaction mixture, made by diluting 2.0 cc. of peptide solution to a total of 3.0 cc. with enzyme and water. The maximum variation between duplicate determinations was equivalent to 2 to 5 per cent hydrolysis of the substrate, depending upon the amount of dissolved materials in the enzyme solution, since these sometimes interfered with the accurate reading of the end-point. In all cases, hydrolysis values have been reported as per cent hydrolysis of one optical component of racemic peptides.

The peptidases of aerobes

Proteus vulgaris. The rate of aeration of the medium in the growth of this organism was very much limited by the excessive frothing which occurred. From 14 liters of medium, 5.1 grams wet cells were obtained. The enzyme solution prepared from these cells (10 hour autolysis) was concentrated *in vacuo* and dialyzed against distilled water for 16 hours. The analysis of the final solution is given in table 2.

Johnson, Johnson and Peterson (1936) found that leucylpeptidase of hog erepsin was activated by Mg ions and that it hydrolyzed LG[3] and LGG at equal rates. Tests for Mg^{++} activation were therefore made with several bacterial enzyme solutions to see if any of these contained enzymes resembling animal leucylpeptidase. The solutions before testing were reduced in Mg content by acetone precipitation. The data of table 2 show that the hydrolysis of LG and LGG by the leucylpeptidase of *Proteus vulgaris* was activated by Mg^{++}. Furthermore, the two peptides were hydrolyzed at approximately the same rate by the crude enzyme solution as well as by the

TABLE 2

Peptidases of Proteus vulgaris

SUBSTRATE	CRUDE ENZYME*			HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME†		RECOVERY OF ENZYME AFTER PRECIPITATION
	Time of incubation	Hydrolysis‡	Yield per 10 grams wet cells	No Mg	0.003 M Mg	
	hours	*per cent*	*cc.*§	*per cent*	*per cent*	*per cent*
dl-AG	1	20	13	25	27	6
dl-LG	3	20	4.3	14	76	48
GG	13	34	1.6	8	5	8
dl-AGG	15	29	1.3	6	7	13
dl-LGG	3	23	4.9	18	73	40
GGG	15	8	0.3	2	2	16

* 0.50 cc. (representing 77 mgm. of wet cells) of crude enzyme was used per determination.

† For demonstration of Mg^{++} activation, 8.0 cc. of crude solution at pH 6.3 were mixed with 16 cc. acetone, let stand 1 minute, and centrifuged. The precipitate was suspended in 8 cc. water, and recentrifuged. 0.50 cc. of the resulting clear solution was used in all cases; incubation was for 24 hours.

‡ Per cent hydrolysis of one optical component of racemic peptides. 0.003 M $MgCl_2$ was present in the substrates.

§ The basis for calculating yields has been described in a previous paper (Berger, Johnson and Peterson (1938)). Briefly stated, the values represent the hydrolysis which would be obtained by an extract from 10 grams wet cells in one hour at 40°, expressed as cc. of 0.2 N HCl.

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[3] In this paper the following abbreviations will be used: LG = leucylglycine, LGG = leucyldiglycine, AG = alanylglycine, AGG = alanyldiglycine, GG = diglycine, GGG = triglycine.

acetone-precipitated preparation, both in the absence and presence of Mg ions.

Pseudomonas fluorescens. 6.8 grams wet cells were obtained from 14 liters of medium. Here too, the rate of aeration was greatly reduced on account of excessive frothing of the medium. The enzyme solution prepared from these cells (10 hour autolysis) was concentrated *in vacuo* and dialyzed 16 hours. The analysis is given in table 3. It may be seen that the hydrolysis of LG, LGG, GGG and possibly AGG was activated by Mg^{++}. It is very interesting to note that the GG- and GGG-splitting enzymes were recovered completely after acetone precipitation, whereas the enzymes of animal erepsin and mold which split these peptides are almost completely destroyed by acetone precipitation at room temperature.

TABLE 3

Peptidases of Pseudomonas fluorescens

SUBSTRATE	CRUDE ENZYME*			HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME†			RECOVERY OF ENZYME AFTER PRECIPITATION
	Time of incubation	Hydrolysis	Yield per 10 grams wet cells	Time of incubation	No Mg	0.003 M Mg	
	hours	*per cent*	*cc.*	*hours*	*per cent*	*per cent*	*per cent*
dl-AG	2	36	8.7	2	20	20	56
dl-LG	2	62	15	8	14	57	23
GG	15	52	1.2	9	28	31	100
dl-AGG	2	30	7.3	8	36	41	34
dl-LGG	2	56	13	8	26	50	22
GGG	15	78	2.9	8	26	40	96

* 0.50 cc. (corresponding to 103 mgm. wet cells) of crude enzyme solution was used per determination. Substrates contained 0.003 M $MgCl_2$.

† For demonstration of Mg^{++} activation, 8 cc. crude solution were mixed with 16 cc. acetone and centrifuged after 3 minutes. The resulting precipitate was suspended in 8 cc. H_2O, recentrifuged and 0.50 cc. of clear solution used per determination.

Basis for calculating yields same as in table 2.

Bacillus subtilis. 15.1 grams wet cells from 14 liters of medium were frozen 6 weeks with intermittent thawing, then autolyzed with toluene for 24 hours. The clear solution obtained after centrifuging was tested for the presence of Mg-activated enzymes. As shown in table 4, the hydrolysis of LG was activated but the lack of activation of LGG hydrolysis was rather surprising since with *Proteus*, *Pseudomonas* and animal leucylpeptidase both LG and LGG hydrolyses were activated. The stability of the GGG-splitting enzymes to acetone precipitation was markedly different from the behavior of the GGG-splitting enzyme in erepsin towards this treatment and resembled that found in *Pseudomonas fluorescens* (table 3). LGG was found to be hydrolyzed by the acetone-precipitated enzyme preparation at a pH optimum of 7.7 as shown in figure 1.

TABLE 4

Peptidases of B. subtilis

SUBSTRATE	CRUDE ENZYME*		HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME†			RECOVERY OF ENZYME AFTER PRECIPITATION
	Time of incubation	Hydrolysis	Time of incubation	No Mg	0.003 M Mg	
	hours	*per cent*	*hours*	*per cent*	*per cent*	*per cent*
dl-AG	6	22				
dl-LG	24	70	24	46	70	100
GG	24	12				
dl-AGG	1	60	1	71	71	118
dl-LGG	1	60	1	59	59	100
GGG	24	70	24	61	49	87

* 1.0 cc. crude enzmye solution (corresponding to 125 mgm. wet cells) was used per determination. The substrates contained 0.003 M $MgCl_2$.

† To test for Mg^{++} activation, 10 cc. crude enzyme solution at pH 6.84 were mixed with 10 cc. of acetone; the precipitate obtained was centrifuged after 3 minutes, then suspended in 10 cc. H_2O, and recentrifuged. 1.0 cc. of the clear solution was used per determination.

Escherichia coli. During the course of this work 100 grams of wet cells were grown aerobically. The average yield was 25 grams from 14 liters of medium (strongly aerated). It was found that with *E. coli* just as with *B. megatherium* the peptidases were not readily extracted from fresh cells by a 17 hour autolysis in 40 per cent glycerol at 37°. (Only 12 units of LG-splitting enzyme were obtained per 10 grams wet cells.) Table 5 gives

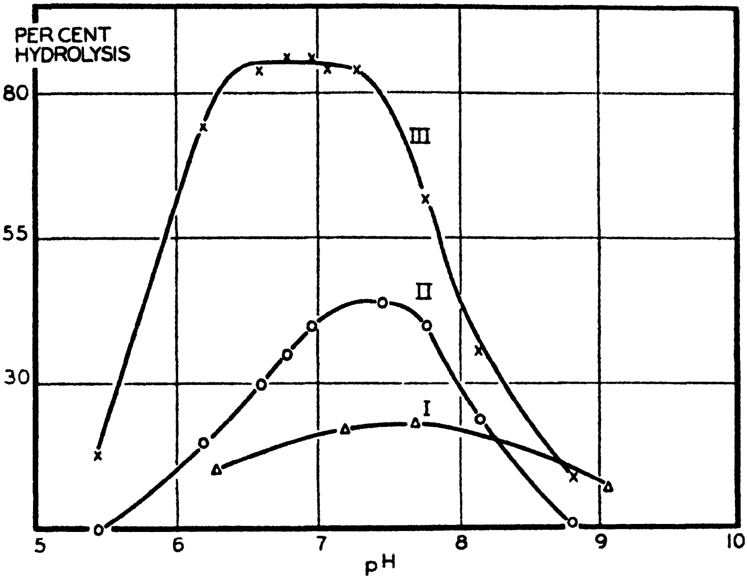

FIG. 1. pH optima for hydrolysis of LGG by *B. subtilis* (*I*, acetone-precipitated enzyme solution, incubation period 1 hour), and *C. sporogenes* (*II*, after 1 hour incubation, *III*, after 4 hours incubation at 40°).

TABLE 5

Peptidases of E. coli

SUBSTRATE	AEROBIC[*]		ANAEROBIC[†]	
	Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	Yield per 10 grams wet cells
	per cent	*cc.*	*per cent*	*cc.*
dl-AG	94	127	24	7.0
dl-LG	41	56	18	5.2
GG	50	68	16	4.7
dl-AGG	67	91	52	15
dl-LGG	34	46	68	39
GGG	40	54	11	2.9

[*] 24 grams of wet cells were frozen 2 days with intermittent thawing, then autolyzed for 4 hours. 0.4 cc. crude enzyme solution, corresponding to 36.9 mgm. wet cells, was used per determination. Incubation time was one hour.

[†] 1.0 cc. crude enzyme solution, corresponding to 171 mgm. wet cells, was used per determination. Incubation time was 1 hour. Substrates contained 0.003 M $MgCl_2$.

Basis for calculating yields same as in table 2.

the results of the analyses on a representative enzyme preparation. The yield of peptidases may be seen to be higher than for any organism yet studied by us with the exception of *Leuconostoc mesenteroides.*

Space will not be taken here to present the experimental data on the kinetics of the peptidases, since they were determined by the use of crude solutions. It was found that at pH 8, the hydrolysis of AG, LG, AGG, LGG and GGG proceeded at a constant rate while the hydrolysis of GG was a first order reaction. That *E. coli* contains a leucylpeptidase was shown by the fact that in the presence of 0.003 M Mg^{++}, the hydrolysis of LGG by an acetone-precipitated enzyme preparation was activated fourfold, namely from 10 to 44 per cent.

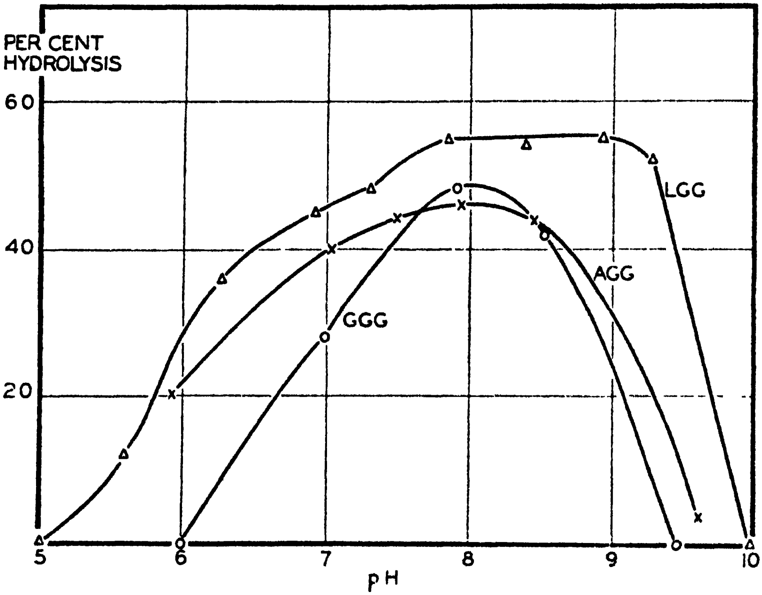

FIG. 2. pH optima for hydrolysis of LGG, AGG and GGG by peptidases of *E. coli*. Incubation time was 1 hour at 40°.

Figures 2 and 3 summarize the data obtained for the optimum pH values for the hydrolysis of peptides by *E. coli* peptidases. From the shape of the curves obtained for LG and LGG hydrolysis, it appears that in both cases a second enzyme with pH optimum at 6 to 7 is involved. The very rapid decrease in the rate of hydrolysis of these two peptides after pH 9.3 and 9.6 is possibly due to the precipitation of the metal activator as $Mg(OH)_2$.

When *E. coli* was grown anaerobically, 8.6 grams wet cells (from 14 liters of medium) were obtained. The frozen cells were autolyzed 24 hours, and the resulting enzyme preparation, after clarification, was analyzed on 6 peptides. The results are given in table 5. When grown anaerobically, the cells appeared to contain less enzyme than when grown aerobically. Furthermore, the leucyl peptides were hydrolyzed more rapidly than the glycyl peptides, a reversal of the picture presented by the aerobically grown cells. A test for Mg-activatable peptidases gave negative results.

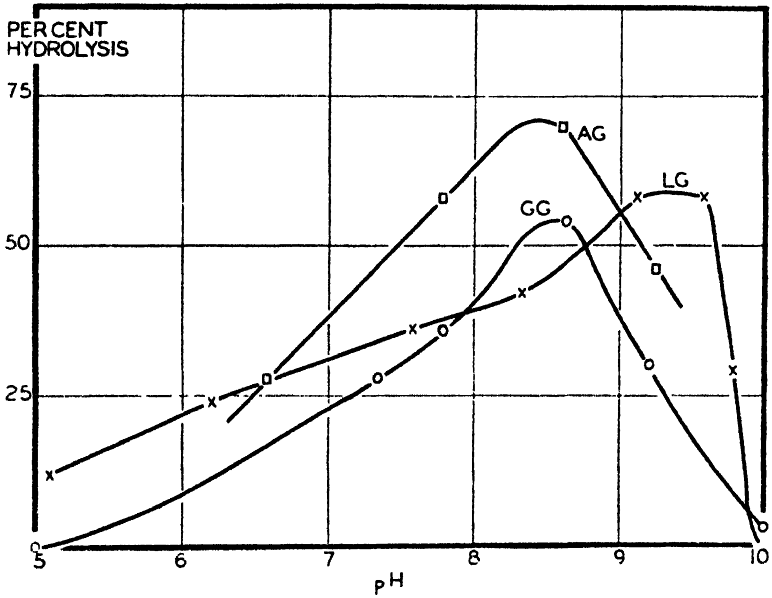

FIG. 3. pH optima for hydrolysis of AG, LG and GG by peptidases of *E. coli*. Incubation time was 1 hour at 40°.

Bacillus megatherium. During the course of this study, 650 grams of wet cells were grown. The average yield was 40 grams wet cells per 14 liters of medium. Table 6 gives the data obtained for analyses on 6 peptides with a representative enzyme preparation. When a crude enzyme solution was used, it was found that the hydrolyses of LG, AG, GG and LGG were first order reactions while GGG and AGG were linear.

TABLE 6

Peptidases of B. megatherium

SUBSTRATE	ENZYME SOLUTION PER DETERMINATION	PREPARATION VIII*			
		Dialyzed enzyme solution†		Acetone-precipitated enzyme‡	Recovery of enzyme after precipitation
		Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	
	cc.	*per cent*	*cc.*	*per cent*	*per cent*
dl-AG	0.1	68	71	30	44
dl-LG	0.3	48	17	20	42
GG	0.3	40	14	14	35
dl-AGG	0.3	54	19	14	26
dl-LGG	0.1	46	48	24	52
GGG	1.0	14	1.5	7	25

* Preparation VIII—62 grams wet cells were frozen and thawed 6 times during 2 days, then autolyzed with toluene for 12 hours. After clarification, the solution was concentrated in vacuo and dialyzed 10 hours.

† 1 cc. dialyzed solution corresponds to 480 mgm. wet cells. Incubation time was 1 hour.

‡ Acetone-precipitation—5 cc. dialyzed enzyme concentrate (pH 6.55) were mixed with 10 cc. acetone. The resulting precipitate was immediately centrifuged off, then suspended in 10 cc. H_2O. All substrates were incubated 2 hours, except GGG (4 hours).

Basis for calculating yields same as in table 2.

Acetone, ethyl alcohol, methyl alcohol, dioxan and combinations of some of these were used as precipitating agents. On the average, 40 to 50 per cent of the enzyme was recovered with acetone (table 6) or dioxan but less than 10 per cent with ethyl or methyl alcohol. When acetone was used to precipitate from a dilute enzyme solution, practically complete recovery of LG-, GG- and LGG-splitting enzymes was obtained. Preliminary experiments on adsorption of enzymes on β-$Fe_2O_3 \cdot H_2O$ in aqueous solution showed that at pH 6.04, from dilute solution, the LGG-splitting enzyme was preferentially adsorbed, leaving behind in the filtrate a purer LG- and GG-splitting solution. $Al(OH)_3$-C_γ also adsorbed the LGG-splitting enzyme preferentially but did not give as sharp a separation.

A number of times enzyme preparations of *B. megatherium* were acetone-precipitated and the resulting preparations tested

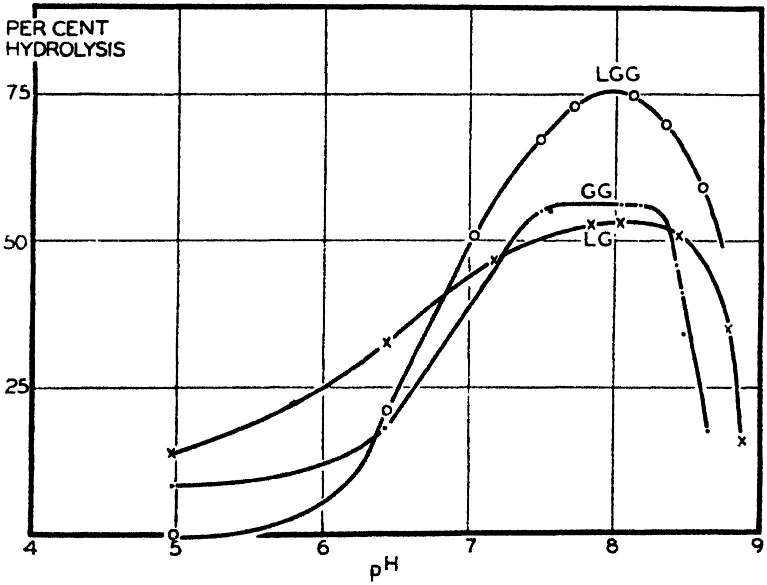

FIG. 4. pH optima for hydrolysis of LG, GG and LGG by peptidases of *B. megatherium*. Incubation time was 1 hour.

for the hydrolysis of the usual 6 peptides in the presence and absence of 0.003 M Mg^{++}. No activation was observed at any time. Another procedure to test for Mg^{++} activation was also tried. An enzyme solution was dialyzed until it had decreased 50 per cent in activity and then was tested on LG and LGG in the presence and absence of Mg^{++}. No activation was observed.

From figure 4 it may be seen that the pH optimum for hydrolysis of GG was 7.6 to 8.0 and of LG and LGG, 8.0.

Phytomonas tumefaciens. This organism was grown on a synthetic medium of the following composition:[4]

Sucrose	10.0 grams
$(NH_4)_2SO_4$	7.5 grams
Glutamic acid	2.5 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
NaCl	0.2 gram
K_2HPO_4	10.0 grams
$CaCl_2$	0.1 gram
Distilled water	1000 cc.

pH was adjusted to 6.8

The culture was grown in 6-liter Pyrex bottles with continuous aeration by sterile, filtered air for 88 hours at 26°. The cells were centrifuged off, frozen two weeks and thawed intermittently at least 5 times. They were then suspended in water, the pH adjusted to 7 and the mixture allowed to autolyze with toluene at room temperature. Samples were analyzed daily for LG- and LGG-splitting enzymes until no further increase appeared

TABLE 7

Peptidases of P. tumefaciens and B. mesentericus

SUBSTRATE	P. TUMEFACIENS*		B. MESENTERICUS†	
	Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	Yield per 10 grams wet cells
	per cent	*cc.*	*per cent*	*cc.*
dl-AG	64	3.1	14	1.9
dl-LG	78	3.7	10	1.4
GG	50	2.4	2	0.3
dl-AGG	26	1.2	34	4.7
dl-LGG	48	2.3	42	5.8
GGG	10	0.5	6	0.8

* 1.0 cc. crude enzyme solution corresponding to 210 mgm. wet cells was used per determination. Incubation time was 5 hours.

† 1.0 cc. dialyzed enzyme solution, corresponding to 180 mgm. wet cells was used per determination. Incubation time was 2 hours.

Basis for calculating yields same as in table 2.

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[4] This medium was devised by J. M. Van Lanen, A. J. Riker and I. L. Baldwin at the University of Wisconsin. It gives a yield of 70 grams wet cells (considerable of which appears to be gum) from 14 liters of medium.

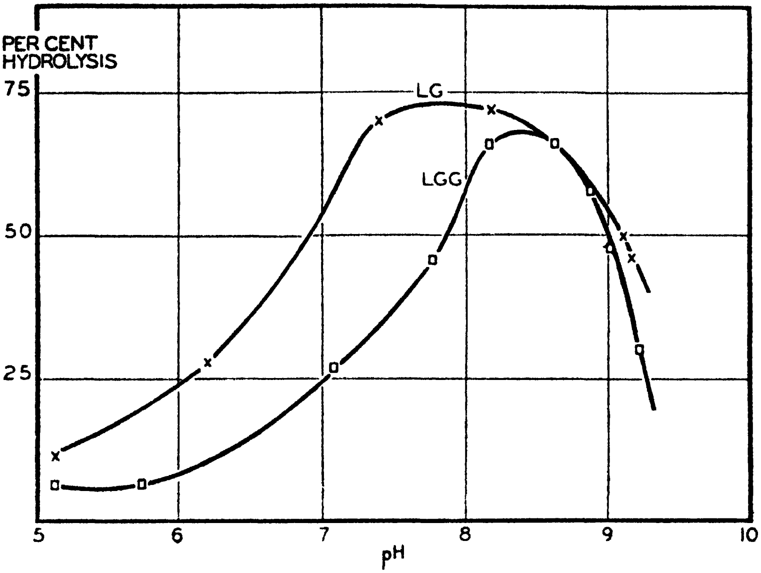

FIG. 5. pH optima for hydrolysis of LG and LGG by peptidases of *P. tumefaciens*. Incubation time was 6 hours at 40°.

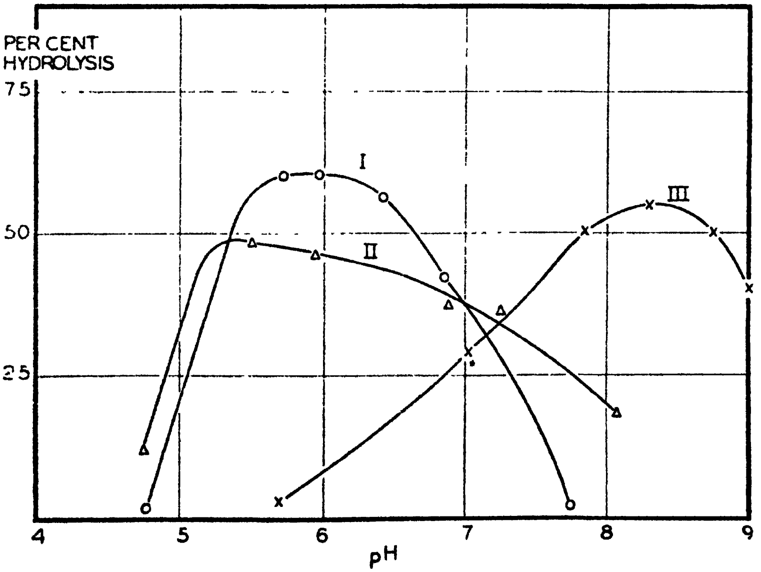

FIG. 6. pH optima for hydrolysis of AG by *P. pentosaceum* (*I*), and of LGG by *L. pentosus* (*II*) and *B. mesentericus* (*III*).

to occur (nine days). The solution was then filtered. Its analysis is summarized in table 7 (which includes also data for *Bacillus mesentericus*). *P. tumefaciens* also contains a Mg- activated leucylpeptidase since the hydrolysis of LGG by an acetone-precipitated enzyme preparation increased from 26 to 44 per cent when 0.003 M Mg^{++} was added. From figure 5 it may be seen that the crude enzyme solution hydrolyzed LG at an optimum pH of 7.6 to 8.2, while LGG was hydrolyzed most rapidly at pH 8.4.

Bacillus mesentericus. 10.6 grams wet cells from 14 liters of medium were frozen 3 months, autolyzed with toluene for 24 hours, and the final enzyme solution dialyzed 14 hours before analysis. The results are given in table 7. It may be seen that leucyl and alanyl peptides were hydrolyzed more rapidly than glycyl peptides, a characteristic common to all the aerobic organisms studied with the exception of *Escherichia coli.* The pH optimum for the hydrolysis of LGG was found to be 8.4, as shown in figure 6.

Specificity of bacterial peptidases

In table 8 are given the data for the hydrolysis of a number of peptides and their derivatives by peptidases of *Escherichia coli* and *Bacillus megatherium.* It may be seen that *E. coli* peptidases hydrolyze glycyl peptides more rapidly than leucyl peptides, while the reverse is true for *B. megatherium.* Substitution of a methyl group for a hydrogen atom of the free amino group such as in sarcosylglycine, N-methylalanyldiglycine and N-methylleucyldiglycine inhibited hydrolysis very markedly with both organisms. *B. megatherium* peptidases, however, hydrolyzed sarcosyldiglycine more rapidly than GGG. Acylated peptides were not split by either organism. For *B. megatherium,* separate determinations (not given in the table) showed that chloroacetylglycine was not hydrolyzed, while prolylglycine and prolyldiglycine were both very slowly hydrolyzed at about 1/50 the rate of LG. Prolylglycine was split 1.7 times as fast as prolyldiglycine. Decarboxylation of peptides such as in leucylmethylamine and glycylmethylamine inhibited hydrolysis by peptidases of both organisms, but *E. coli* peptidases split both compounds very slowly. Yeast dipeptidase and presumably ereptic dipeptidase require a free carboxyl group on the dipeptide to be hydrolyzed (Bergmann *et al.*, 1935), but leucylpeptidase (Johnson *et al.*, 1936) is able to split decarboxylated dipeptides. Since *E. coli* contains an LGG-splitting enzyme whose activity is accelerated by Mg^{++}, and since decarboxylated dipeptides are hydrolyzed by the crude enzyme solution, the presence of an animal-leucylpeptidase-like enzyme is strongly suggested.

TABLE 8

Hydrolysis of synthetic peptides by E. coli and B. megatherium peptidases

Data are expressed as per cent hydrolysis of one optical component of racemic peptides. Thus 200 per cent splitting of AGG means that 2 linkages (presumably of the natural form of the peptide) have been completely hydrolyzed with the formation of 3 amino acid molecules.

SUBSTRATE	E. COLI (AEROBIC PREP. II)*			B. MEGATHERIUM (PREP. VII)†		
	Per cent hydrolysis of one linkage					
	1 hour	6 hours	24 hours	1 hour	6 hours	24 hours
dl-AG	94	100	107	41	88	94
dl-LG	41	100	101	13	47	83
dl-Leucylmethylamine	4	9	12	1	5	5
GG	50	84	90	9	31	49
Glycylmethylamine	2	4	12	0	0	0
Sarcosylglycine	0	5	23	0	0	3
Sarcosyl-*l*-tyrosine	0	2	4	0	1	10
dl-AGG	67	184	201	11	47	138
dl-N-Methylalanyldiglycine	4	20	58	4	5	65
dl-LGG	34	162	202	36	102	136
dl-N-Methylleucyldiglycine	5	13	83	1	1	7
GGG	40	140	183	0	1	18
Sarcosyldiglycine	6	29	169	1	3	35
Benzoyldiglycine	0	1	1	0	0	0
Tetraglycine	33	164	272	2	4	16
Chloroacetyl-*l*-tyrosine	0	0	1	1	1	6

* 0.40 cc. crude enzyme solution (corresponding to 36.9 mgm. wet cells) was used per 3 cc. of reaction mixture in all determinations.

† 0.30 crude enzyme solution (corresponding to 50 mgm. wet cells) was used per 3 cc. of reaction mixture in all determinations.

An activation appears to occur in the hydrolysis of N-methylleucyldiglycine and sarcosyldiglycine between 6 and 24 hours of incubation (*E. coli*) and in the hydrolysis of N-methylalanyldiglycine and sarcosyldiglycine by *B. megatherium* peptidases. The reason for this is not clear.

The peptidases of anaerobes

In the investigation of the 7 aerobes, the characteristics of the peptidases did not appear to differ markedly from the properties of known peptidases. The enzymes hydrolyzed their peptide substrates at pH optima close to pH 8, and were reasonably stable on incubation at this pH value and 40°. There was no particular reason to suspect that a procedure different from the one used would give better results. However, in the study of 5 anaerobes, differences, apparently in the nature of the peptidases, soon became obvious. With further investigation, the number of these differences increased until it was realized that anaerobes contained peptidases which could not be studied properly under the same conditions that were used for the aerobes. These differences in stability, pH optima and activation by reducing agents will be pointed out for the organisms where they appeared.

Clostridium sporogenes and *Closteridium butylicum.* 11.8 grams wet cells of *C. sporogenes* (from 14 liters of medium) were frozen 1 week, thawed 5 times and autolyzed 10 hours. 18.6 grams wet cells of *C. butylicum* grown on a 0.5 per cent tryptone, 0.5 per cent glucose medium, were frozen 3 months, then autolyzed for 79 hours. The analyses of these enzyme solutions are given in table 9 (which also includes data for *Clostridium acetobutylicum*). When cells of *C. butylicum* grown on the regular peptone-glucose medium were frozen 1 week and autolyzed 10 hours, 2.7 and 8.5 units of AGG- and LGG-hydrolyzing enzymes respectively per 10 grams wet cells were obtained. In an incubation period of 2 hours, no hydrolysis of the other 4 peptides tested was obtained.

We noticed in our work a marked instability of the peptidases of these two organisms and also of certain other anaerobes. In a previous paper (Berger, Johnson and Peterson, 1938) it was shown that the unstable AGG-hydrolyzing enzyme of *Leuconostoc mesenteroides* was activated by a large number of heavy metals. It has subsequently been found that reducing agents such as thioglycolic acid, cysteine, H_2S, HCN and monomethyl-paramido phenol sulfate ("metol") also have a marked activating effect on this peptide hydrolysis. Data are given in table 10. The AGG-hydrolyzing peptidases of *C. butylicum* (and *Lactobacillus pentosus*) were also activated by "metol." Investigations at present under way indicate that the peptidases of many anaerobic species are activated by reducing agents. Weil and Kocholaty (1937) and Maschmann (1938) have reported activation of bacterial proteinases by reducing agents, but did not mention activation of peptidases.

TABLE 9

Peptidases of C. sporogenes, C. butylicum and C. acetobutylicum

SUBSTRATE	C. SPOROGENES*		C. BUTYLICUM†		C. ACETOBUTYLICUM‡		
	Hydrolysis	Yield per 10 grams wet cells	Hydrolysis	Yield per 10 grams wet cells	Time of incubation	Hydrolysis	Yield per 10 grams wet cells
	per cent	*cc.*	*per cent*	*cc.*	*hours*	*per cent*	*cc.*
dl-AG	2	0	22	3.9	24	30	0.5
dl-LG	1	0	22	3.9	24	26	0.5
GG	1	0	6	1.1	24	6	0.1
dl-AGG	16	4.9	14	2.5	6	18	1.3
dl-LGG	50	15	44	7.8	6	14	1.0
GGG	2	0	10	1.8	24	6	0.1

* *C. sporogenes*—Substrates contained 0.003 M $MgCl_2$. 0.5 cc. crude enzyme solution (corresponding to 82 mgm. wet cells) was used per determination. Time of incubation was 2 hours, except for GGG (4 hours)

† *C. butylicum* 0.75 cc. crude enzyme solution (corresponding to 140 mgm. wet cells) was used per determination. Incubation time was 2 hours throughout.

‡ *C. acetobutylicum*—Substrates contained 0.003 M $MgCl_2$. 1.0 cc. of crude enzyme solution (corresponding to 115 mgm. wet cells) was used per determination.

Basis for calculating yields same as in table 2.

The pH optimum for the hydrolysis of LGG by *C. sporogenes* peptidases was found to be 7.4 as shown in figure 1. When determinations were made after a 4-hour incubation period instead of 1 hour, the pH optimum appeared to be 6.5 to 7.2. This, however, is probably the pH stability range. These data suggest that only determinations in which relatively short periods of incubation are used can be relied upon to give true pH activity optima. In the literature review in table 1, 24 hours was the shortest incubation period used in the determination of any of the pH optima. We have found the pH optimum for the hydrolysis of AGG in the presence of 10^{-3} molar "metol" by *C. butylicum* peptidases to be 7.5 (1 hour incubation at 40°).

The hydrolysis of AGG and LGG by an acetone-precipitated enzyme preparation from *C. sporogenes* was not activated by 0.003 M Mg^{++} at pH 8. Thirty-one per cent of the LGG-splitting enzyme was recovered after the acetone precipitation.

TABLE 10

Activation of peptidases by reducing agents (L. mesenteroides)

MOLAR CONCENTRATION OF ACTIVATOR	PER CENT HYDROLYSIS OF AGG				
	Cysteine	H_2S	HCN	Thioglycolic acid	"Metol"
None	27	27	27	27	34
10^{-2}	104	66	66	73	92
10^{-3}	29	44	54	106	94
10^{-4}	29	79	42	30	96
10^{-5}	29	88	36		71

The same enzyme solution gave 80 per cent hydrolysis of AGG in the presence of 10^{-3} M Zn^{++}.

Incubation was for one hour.

Clostridium acetobutylicum. 13.8 grams wet cells from 14 liters of medium were frozen 6 weeks with intermittent thawing, then autolyzed with toluene for 24 hours. The data obtained on the analysis of the enzyme solution are contained in table 9. The low yields of enzyme are possibly due to the inefficiency of the extraction method as well as to the instability of the peptidases in the absence of suitable activating agents.

Lactobacillus pentosus and *Propionibacterium pentosaceum.* 26.7 grams wet cells of *Propionibacterium* and 10.9 grams wet cells of *Lactobacillus* were obtained per 18 liters of a medium which contained in addition to the usual components, 0.5 per cent of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) and 100 cc. of yeast water[5] per liter of medium. The pH of this medium at the time of inoculation was about 5.8. Cells of both organisms were frozen 3 months with intermittent thawing, then autolyzed with toluene for 24 hours. The enzyme solutions were dialyzed 10 hours. The analyses of these solutions are given in table 11. Unfortunately, these data do not give the true picture of the distribution of the peptidases in these organisms since the pH optima for the hydrolysis of AG by *Propionibacterium* and of LGG by *Lactobacillus* were found to be 5.8 and 5.5, as shown in figure 6,

TABLE 11

Peptidases of Lactobacillus and Propionibacterium

SUBSTRATE	L. PENTOSUS	P. PENTOSACEUM
	Hydrolysis	
	per cent	*per cent*
dl-AG	6	20
dl-LG	10	14
GG	6	10
dl-AGG	6	6
dl-LGG	3	0
GGG	0	2

Time of incubation was 2 hours.

1.0 cc. dialyzed enzyme solution was used in each determination, corresponding to 188 mgm. wet cells of *Lactobacillus*, or 534 mgm. for *Propionibacterium*.

and not pH 8 at which value the routine determinations were made. *Propionibacterium* and *Lactobacillus* thus appear to contain acidopeptidases similar to those reported in acidoproteolytes by Gorbach (1937).

Enzymes in the culture medium

Considerable controversy exists concerning the secretion of proteinases and peptidases into the medium by living bacterial cells. Gorbach and Pirch (1936) have claimed that the secretion of proteinase occurs only after partial cell autolysis while Virtanen and Suolahti (1937) insist that living cells secrete proteinase. Whatever the true situation may be, we have found peptidase activity in filtrates from 24- to 48-hour cultures of proteolytic as well as non-proteolytic organisms. This is shown in table 12 from which it may be seen that filtrates of *Escherichia coli* (non-proteolytic) and *Bacillus megatherium* (proteolytic) contained very considerable amounts of peptidases. Filtrates of cultures of *Clostridium sporogenes*, *Clostridium butylicum*, *Clostridium acetobutylicum*, *Proteus vulgaris*, *Bacillus subtilis* and *Bacillus mesentericus* did not contain sufficient LG- or LGG-splitting peptidases to give increases in titration in 10- to 24-hour incubation periods that were significantly greater than experimental error. Cells of these organisms, if analyzed for in the same proportional dilution, would also have given no significant titration, so it is impossible to conclude in these cases whether cells or medium contained more enzyme.

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[5] The clear extract from 200 grams pressed yeast autoclaved in 1 liter of water.

TABLE 12

Peptidases found in culture media

ORGANISM	PREPARATION	SUBSTRATE	TIME OF INCUBATION	HYDROLYSIS	APPROX. YIELD FRO 14 LITERS MEDIUM	
					Medium	Cells*
			hours	*per cent*	*cc.*	*cc.*
B. megatherium† . .	I	GG	48	26	38	56
	II	*dl*-LGG	6	24	280	192
		dl-LGG	4	27	472	192
	III	*dl*-LG	10	54	378	68
		dl-LGG	5	52	728	192
		GGG	10	8	56	6
E. coli‡		GG	24	26	23	170
		dl-LGG	18	56	65	115

* Values for enzyme yields from cells were taken from tables 5 and 6. 25 grams wet cells of *E. coli* and 40 grams wet cells of *B. megatherium* were taken as average cell yields per 14 l. medium.

† 1 cc. filtrate of 24 hour cultures was used per determination.

‡ 3 liters of filtrate from a 45 hour culture were concentrated in vacuo (below 40°) to 900 cc.: this was dialyzed 18 hours, then 1 cc. used per determination.

Basis for calculating yields same as in table 2.

DISCUSSION

In a wide survey of this kind, optimum conditions for analysis of all the organisms could not be maintained. For the aerobes, the general procedure adopted proved to be very satisfactory. When the anaerobes were subjected to this procedure, however, it was found that their peptidases had properties differing very considerably from those of any previously studied peptidases. The instability under the experimental conditions employed, the activation by reducing agents and heavy metals, and the acidic pH optima of some of the peptidases greatly increased the difficulties involved in their analysis. This study has outlined the general properties of a large number of bacterial peptidases and also suggested the peculiar properties possessed by some peptidases. These characteristics must be kept in mind in a detailed investigation of any one species of bacteria.

SUMMARY

1. A survey has been made of the peptidases in cell-free extracts of twelve bacterial species, including *Escherichia coli*, *Bacillus megatherium*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus mesentericus*, *Bacillus subtilis*, *Clostridium butylicum*, *Clostridium sporogenes*, *Clostridium acetobutylicum*, *Lactobacillus pentosus*, *Propionibacterium pentosaceum* and *Phytomonas tumefaciens*.

2. An extraction procedure has been found, involving repeated freezing and thawing of cells, followed by a toluene autolysis, which gives satisfactory enzyme preparations with a degree of activity which is measurable within short periods of time.

3. The pH optima have been determined for the hydrolysis of AG by 2 organisms, LG by 3 organisms, GG by 2, AGG by 2, LGG by 7 and GGG by 1. In most cases the optimum value for peptide hydrolysis was between pH 8 and 9. Two organisms, however, *Lactobacillus pentosus* and *Propionibacterium pentosaceum* were found to contain acidopeptidases which split their substrates at optimum pH values of 5.5 to 6.0.

4. In most cases the peptidases were quite stable at pH 8

and 40° but the LGG-splitting enzymes of the anaerobes (*Clostridium sporogenes, Clostridium butylicum*) were very unstable under these conditions. This instability was partly overcome by the addition of reducing agents, which for *Clostridium butylicum* activated the hydrolysis of AGG and LGG.

5. The specificity of the peptidase systems of *Bacillus megatherium* and *Escherichia coli* was studied in some detail. Dipeptides and tripeptides were readily hydrolyzed but acylated or decarboxylated peptides were hydrolyzed only very slowly or not at all. Substitution of a methyl group for a hydrogen atom of the free amino group on a peptide resulted in a very marked decrease in hydrolysis.

6. In four organisms, namely *Escherichia coli, Proteus vulgaris, Pseudomonas fluorescens* and *Phytomonas tumefaciens* a leucylpeptidase-like enzyme was found whose hydrolysis of LGG was activated by 0.003 M Mg^{++}.

7. Appreciable amounts of peptidases were found in culture filtrates of *Escherichia coli* and *Bacillus megatherium*. With *Escherichia coli*, more enzymes could be extracted from the cells than from the medium on which they were grown, as might be expected with a non-proteolytic organism. With the proteolytic organism *Bacillus megatherium*, more peptidases were consistently found in a given volume of medium than could be obtained from the cells grown on the same volume of medium.

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A NEW METHOD FOR THE DETERMINATION OF DISINFECTION RATES

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Received for publication July 1, 1938

In the examination of disinfectants, the rate of killing of bacteria is implicitly contained in the result, even when that result is expressed in any such standard form as the phenol coefficient. This fact is not apparent and is not generally recognized. Let it be recalled, however, that the time to kill is the basic determination upon which the coefficient, or any other relative measure of efficiencies, is erected. "Time to kill" is related to "rate of travel," although in the killing reaction the relation is not quite so simple. It is defined by the value k in the monomolecular reaction formula

$$k = \frac{1}{t} \log \frac{I}{S}$$

where t = the time of contact between disinfectant and organism,

I = the number of organisms at the beginning of the exposure,

S = the number of survivors at the end of the exposure.

With many materials in common use, the rate is so rapid that the usual experimental techniques fail to yield any survivors (no numerical value of S) so that neither the value of k nor any "end-point" can be determined. In common practice an attempt is made to overcome the difficulty by dilution of the disinfectant, since, in general, the greater the dilution the slower is the disinfectant action. In the widely-used phenol coefficient technique a predetermined rate of disinfection is chosen and the dilution at which this rate exists is found by experiment. The difficulties introduced by the use of dilution, however, are many, involving differences between one disinfectant and another as well as variability, with a given disinfectant, in the rate of change of *k* with concentration. An inspection of the equation of rate shows that if *k* is large the experimenter faces the necessity of making the time of contact very small or the initial number of organisms very large. Because of the peculiar nature of the formula which involves the logarithm of the initial number of organisms but not the logarithm of the time, the choice, as will be demonstrated below, is limited to a manipulation of the time variable. With this in mind, an apparatus has been devised in which the time of contact between disinfectant and organism may be made as short as 0.11 second which is less than one one-hundreth of the period of contact obtainable with the methods in use up to the present time.

The reason for the choice of a small value for *t* rather than the use of large initial numbers of organisms may be made clear by the substitution of numerical values in the equation of rate. If *k* is assumed to be 300 for a one-minute technique, the initial number of organisms, in order to have 1 survivor, would have to be 1×10^{300} organisms. This number of organisms is beyond all human comprehension. In a closely packed condition, such a mass of bacteria would fill our universe billions and billions of times. If the technique is so designed that the unit of exposure becomes 0.1 minute or 6 seconds, the initial number of organisms would have to be 1×10^{30} organisms per unit volume. This is also beyond the range of practical conception. If the time of exposure is again reduced, let us say to 0.6 second or to one-tenth of the last value, the initial number required for a survival of 1 organism becomes 1×10^{3} power or 1,000 organisms, a number which is easily obtainable and which in addition, may be increased considerably, permitting the survival of a larger number of organisms and a consequent increase in the reliability of the results. With the apparatus to be described, the short contacts between organisms and disinfectant allow the use of small volumes of materials and the determination of values of

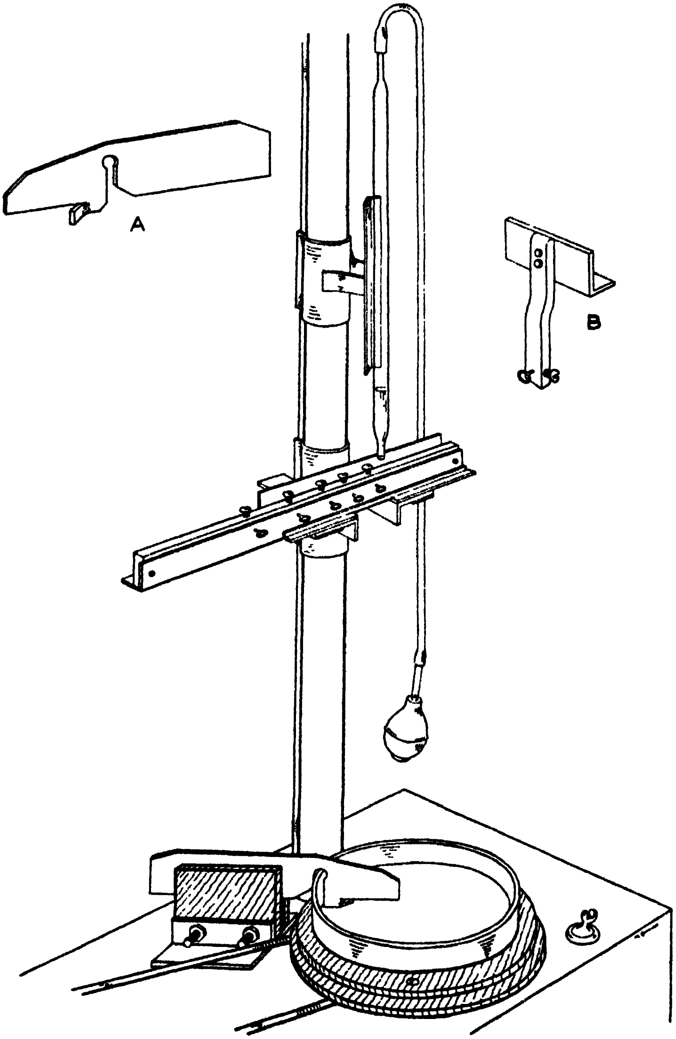

Fig. 1

k, even in excess of the figure used in the calculations made above.

APPARATUS

The apparatus[1] consists essentially of a pipette from which a drop of disinfectant may be released, a platinum loop placed in the path of the falling drop and below these, on a revolving turntable a petri dish containing water or a solution which arrests the action of the disinfectant. The dish is provided with a baffle which aids in the mixing of the drop, containing disinfectant and culture, with the neutralizing agent.

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[1] The apparatus may be obtained from J. H. Emerson, Cambridge, Mass.

Considered in more detail the parts are:

1. *The pipette for disinfectant.* This may be an ordinary 10 cc. Mohr pipette, but the tip must be broken off or ground so that the drop emerges from an opening of about 2 mm. in diameter. The drop is released by gentle pressure on a rubber bulb connected to the pipette by means of a piece of rubber tubing. The volume of the drop is approximately 0.07 cc. When the disinfectant is subsequently mixed with the culture in the loop, it is diluted to the extent of 1:1.07.

2. *The platinum loops.* In the apparatus pictured, 5 of these are mounted in a removable loop carrier. The loops have an inside diameter of 2.5 mm. and are made of 22 gauge wire. The loop carrier has a series of notches (not shown in the diagram) which engage a spring on its holder, thus permitting the accurate centering of each loop beneath the tip of the pipette. The holder of the loop carrier is adjustable both vertically and horizontally. It is adjusted in the usual set-up so that the loops are 15 cm. above the surface of the liquid and 2.5 cm. below the tip of the pipette.

3. *The petri dish, turntable and baffle.* Pyrex dishes (15 mm. depth) have been found to be most uniform.

The structure of the baffle is illustrated in the figure. It is placed at an angle of 22½ degrees from a line running through the center of the upright and the turntable. It serves to counteract the effect of the centrifugal force on the water and facilitate the mixing.

The turntable is so placed that the falling drop hits the surface of the liquid about 2 to 3 cm. from the side of the dish. It revolves clockwise at a speed of approximately 100 r.p.m. At a speed much below this figure, the mixing is not satisfactory, while at higher speeds other difficulties may be encountered.

4. *Pipette for organisms.* In addition to the apparatus shown in the figure there is needed a 0.1 cc. pipette graduated in hun-

dreths of a cubic centimeter. The graduations should be about 1.5 cm. apart. This pipette is used to charge loops.

PROCEDURE IN MAKING A TEST

In carrying out a test the procedure is as follows. A sterile petri dish containing 15 cc. of water or other neutralizing agent (thiosulphate if iodine is being tested, etc.) is put on the turntable and the baffle, which has previously been sterilized by flaming, is put into place. Disinfectant is put in the pipette. The loops are next flamed and each charged with 0.005 cc. of suspension of organisms. This is done by allowing 0.005 cc. of suspension to form a drop on the tip of the 0.1 cc. pipette. The drop is transferred to the loop by contact. The first loop is brought into position under the pipette and a drop of disinfectant is released from the latter by a gentle pressure on the rubber bulb. The next loop is then placed in position and a second drop of disinfectant allowed to fall, this process being repeated as many times as desired. The limit in any case is a concentration of disinfectant strong enough to act as an inhibiting agent later on, or one which may continue the disinfectant action in the dish. The contents of the dish are plated out in agar in suitable quantities or dilutions so that on incubation a count may be made of the number of organisms surviving the action of the disinfectant under test.

The initial number of organisms may be determined best by the use of the technique just described except that water or broth is placed in the pipette instead of the disinfectant.

After the plates are incubated and counted the results may be expressed in terms of a percentage survival and comparisons between different disinfectants may be made on this basis. Since the apparatus as described makes no provision for temperature control, it is necessary to work in a space in which the temperature is reasonably constant.

DETERMINATION OF k VALUES

While the procedure just described enables one to determine a percentage survival under a specified set of conditions, one more value is needed,—the time of contact,—if the equation given in the first paragraph is to be solved for *k*. The well-known formula for falling bodies, $s = \frac{1}{2} gt^2$, gives the time required for the drop to fall from the pipette to the dish as well as that required for the fall from the pipette to the loop, the difference being the time for the fall from the loop to the dish. This calculated value however, is not the value of *t* in the equation, for it must be remembered that the action of the disinfectant continues while it is undergoing dilution. At the instant the drop hits the liquid in the dish the disinfectant action is at its maximum but as mixing and dilution proceed the action falls until it becomes so slow that it may be neglected. The value of *t* needed for the equation must sum up the whole disinfectant action in terms of undiluted disinfectant. This may be called the "equivalent" time of mixing.

The determination of this hypothetical time is fortunately not difficult, the method of calculation being seen most easily in a description of an actual experiment and the use of the data obtained from it. First, a determination of the survival after a 15 cm. fall, according to the method already described, was made, with 5 per cent phenol and *Staphylococcus aureus*. Next an experiment was run in which the special loop holder (*B* in the figure) was used. The loop was placed about 8 mm. from the surface of the liquid, and the tip of the pipette about 8 cm. above the loop. The purpose of this arrangement was to make the time of contact between organism and disinfectant in the air a negligible quantity, so that any organisms killed might safely be assumed to have been acted upon during the process of dilution of the disinfectant in the dish. The data obtained were as follows:

Initial number of organisms	3,200
Survivors after 15 cm. drop	680
Survivors after immediate mixing	1,600

It will be seen that the process of mixing alone leaves 50.0 per cent of the organisms surviving. In other words it may be inferred that the 680 organisms surviving from the 15 cm. fall represent only 50.0 per cent of the organisms which were living at the instant when the drop of disinfectant and culture hit the surface of the water in the dish. This number was therefore 2 × 680 or 1360 organisms. Since the calculated time for falling 15 cm. (after the drop has already fallen 2.0 cm.) is 0.122 second,[2] we may proceed to calculate the k of the reaction. Substituting in the formula we have

$$k = \frac{1}{0.122} \log \frac{3200}{1360}$$

$$k_{(\text{sec.})} = 3.04$$

Now, having the value of k, we may calculate t, the equivalent time of mixing, by substituting the proper values in the same basic equation, which gives,

$$3.04 = \frac{1}{t} \log \frac{3200}{1600}$$

$$t = 0.099 \text{ sec.}$$

The actual time of mixing therefore is equivalent to 0.099 second exposure to full-strength disinfectant. A series of eight experi-

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[2] For convenience in estimating the time of fall the following solutions of the equation $s = \frac{1}{2}gt^2$ are given.

s	TIME (t)	s	TIME (t)
cm.	*second*	*cm.*	*second*
0.5	0.032	15	0.174
1.0	0.045	16	0.181
1.5	0.055	17	0.186
2.0	0.064	20	0.202
2.5	0.071	25	0.226
3.0	0.078	30	0.247
4.0	0.090	31	0.251
5.0	0.101	32	0.255
10.0	0.142		

As an illustration of the use of the table let us consider the following set-up. The tip of the pipette is placed 2.5 cm. from the loop. The loop is 15 cm. above the liquid in the receiving dish, the diameter of the drop emerging from the pipette is 0.5 cm., the drop therefore falls 2 cm. to the loop and 15 cm. to the dish making a total of 17 cm. fall. Referring to the table, the time required to fall 17 cm. is 0.186 second. The time required to fall the 2 cm. is 0.064 second. During the last mentioned period of time, the drop of culture is falling unmixed with disinfectant and therefore must be subtracted from the total giving us 0.122 second. When this figure is added to the mixing time of 0.11 second, the total time of contact, in this case 0.23 second, is obtained.

ments gave the following values of t, 0.088, 0.099, 0.100, 0.107, 0.113, 0.114, 0.114, 0.140, the average value being close to 0.11 sec. If then, the apparatus is set up as described with a 15 cm. fall (the drop first falling 2.0 cm. measured from the bottom of the drop) the total contact of disinfectant and organism is 0.122 + 0.110 second or approximately 0.23 second. The time of mixing may be expected to vary somewhat with the rate at which the disinfectant is inactivated by dilution, the concentration of the disinfectant and nature of the neutralizing medium in the receiving dish.

TESTS OF ACCURACY AND REPRODUCIBILITY OF RESULTS

In calculating the time of contact from the formula $s = \frac{1}{2} gt^2$ the assumption was made that mixture takes place completely and instantaneously when the drop hits the culture contained in the loop. This need not necessarily be true, for it is conceivable that the drop of disinfectant might push the culture ahead of it and mixture might occur only after some distance of fall. It was therefore necessary to test this point experimentally and for this purpose a chemical test was devised. Water was placed in the pipette and 0.005 cc. of normal hydrochloric acid was placed in the loop. Ten drops of water were allowed to fall, as in the bacteriological technique, on ten loops of acid and the acid remaining on the loops and that falling into the dish was titrated. Titration was carried out with N/100 sodium hydroxide. Theoretically, if perfect mixing had occurred during the passage of the water drop through the loop, the concentration of acid in the drop left in the loop should have been the same as the concentration of the acid in the drop which fell into the dish. In a series of nine determinations the average concentration of acid for the drop in the loop was 0.077 N, while that of the drop which continued toward the dish was 0.067 N. These figures indicate that a satisfactory mixing of disinfectant and culture occurs during the extremely short time required for the disinfectant to pass through the loop and therefore the time of contact may be assumed to begin at the instant the drop leaves the loop.

A number of factors are involved in the reproducibility of results. The most important of these are, the constancy of resistance of the organism used, the homogeneity of the suspension (absence of clumps) and the error inherent in the plate count. With one suspension of a filtered culture of *Staphylococcus aureus* diluted in extract broth, the results given in table 1 were obtained. The number of survivors (average of two duplicate plates for each experiment) varied from 57 to 70 with an over-all average of 64. All of these variations are well within the error of the plate count. The variation in cultures made from day to day for the same organism in the same medium is much more marked than that given in the table above. For the organism used in the experiments outlined above the average *k* values for individual broth cultures have run as low as 2.5 and as high as 4.

TABLE 1

Action of 5 per cent phenol on Staphylococcus aureus

INITIAL NUMBER	NUMBER OF SURVIVORS (AVERAGE OF 2 PLATES)	PER CENT SURVIVAL	*k*
			seconds
260	57	21.9	2.87
	64	24.6	2.65
	64	24.6	2.65
	66	25.4	2.59
	70	26.9	2.48

APPLICABILITY OF THE MONOMOLECULAR FORMULA

In devising the apparatus, the applicability of the monomolecular reaction formula was assumed. There are a number of instances reported in the literature, however, where different orders of reaction have been found to prevail and in some cases the reaction rate appears to follow some special formula which probably includes, besides the number of organisms and concentration of disinfectant, a penetration factor. Furthermore, a number of workers have been of the belief that the formula has only an apparent application and that, if shorter times of contact were possible, there would be demonstrated a short period during which the reaction follows a different path.

In order to test whether a reaction is of the first order, that is, of the monomolecular type, two criteria are available. The first involves the constancy of *k* with variation in the initial number of molecules or organisms, the second the constancy of *k* with variation in time of exposure. Experiments were performed to test each of these criteria and the results obtained, while open individually to certain objections, pointed out below, indicate that a reaction of the first order takes place in the time of contact used in the apparatus.

In order to test the first criterion, a series of dilutions of culture was made in broth and each tested out against 5 per cent phenol in the apparatus. The results of a typical experiment are given in table 2. The variations in the per cent survival (20.8 to 25.4), and the corresponding *k*'s (which varied from 2.58 to 3.12) are close enough together to warrant the conclusion that the rate is independent of the initial number of organisms. From this result the further conclusion may be drawn that the reaction of phenol and staphylococcus is one of the first order, provided of course, that the premise is granted that a reaction is occurring which has a rate which is either mono-, bi- or tri-molecular.

TABLE 2

Results of experiments with different initial numbers of organisms

INITIAL NUMBER OF ORGANISMS	FINAL NUMBER	PER CENT SURVIVAL	*k*
			seconds
260,000	66,000	25.4	2.58
26,000	5,000	19.2	3.12
2,600	540	20.8	2.96
260	60	23.1	2.76

The second criterion, which involves experimentally the variation of time of contact, while representing a better test, is difficult to apply since the limitations of the apparatus do not permit a very great range of times of exposure. By permitting a drop to fall 1 cm. before hitting the loop and the mixture of disinfectant and organisms to fall 30 cm. before being neutralized in the dish, a time of contact in the air of 0.21 second was obtained which, together with the equivalent mixing time of 0.11 second, yielded a total exposure of 0.32 second. In a second experiment the drop was permitted to fall 2 cm. and a further 15 cm. after hitting the loop. The times of contact were 0.12 in the air and 0.11 in the dish making a total of 0.23 second. With 4100 organisms as the initial number, the survivors were respectively 205 and 500 organisms, the k values being approximately 3.9 in each case. The difference in time of contact, while apparently not very large, was yet sufficiently great to yield very definite differences in the number of survivors. The conclusion may be drawn that the reaction between phenol and staphylococcus proceeds at a monomolecular rate over a period of time. The results also strengthen the correctness of the assumptions made in the calculation of the equivalent mixing time.

In the interpretation of the results as a whole, a word of caution may be in place. While it has been demonstrated above that in the general neighborhood of 0.2 second contact, the reaction between phenol and staphylococcus proceeds as a monomolecular reaction, there may still be a very short period of contact, perhaps in the nature of 0.01 of a second, during which some other, or perhaps even no action at all, takes place. Secondly, in our use of 1 second as the unit of time there is implied the assumption that the reaction proceeds with unvarying rate for a period at least 5 times longer than that under which the actual experiments have been carried out. Such an assumption need not necessarily be correct since it is conceivable that the reaction rate might change suddenly after a given period of time. Observations of this sort are common in survival curves run with the usual techniques. Finally, the relationships demonstrated between the staphylococcus and phenol may not be carried over without experimental proof to other organisms and other disinfectants. That the reaction between staphylococcus and phenol, however, is not unique has been shown by results obtained with iodine and *Escherichia coli* to be reported later.

SUMMARY

An apparatus for the study of the disinfection process is described, in which contact between organism and disinfectant may be made for periods of about 0.2 second. The set-up permits the determination of disinfection rates of strong disinfectants without appreciable dilution.

HETEROAUXIN AND THE GROWTH OF ESCHERICHIA COLI

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Received for publication July 3, 1938

The possibility that heteroauxin (3-indoleacetic acid) which is produced by bacteria may have a stimulating effect upon the growth of the organism producing it apparently has not been investigated. *Escherichia coli* was chosen for an investigation of this problem since it has been found to produce heteroauxin under appropriate conditions (Hopkins and Cole, 1903).

E. Salkowski and H. Salkowski (1880) and E. Salkowski (1885, 1885a), working with mixed cultures in the putrefaction of proteins, found that 3-indoleacetic acid was associated with the growth of bacteria. *E. coli* produces 3-indoleacetic acid in addition to indol by the breakdown of tryptophane (Hopkins and Cole, 1903). Herter (1908) demonstrated that 3-indoleacetic acid found in the urine of patients came from bacteria that were active in the putrefaction of intestinal contents. This acid was absorbed through the intestinal wall and excreted by the kidneys. Herter and Ten Broek (1909) found that *Proteus vulgaris* produced 3-indoleacetic acid and indol from a peptone-beef extract medium.

Frieber (1921) reported that heteroauxin is produced during the fermentation of tryptophane by both indol-positive and indol-negative bacteria. The breakdown of tryptophane to indol occurs in two stages. During the first stage, 3-indoleacetic acid is produced and in the second stage this acid is converted into indol. Indol-positive bacteria, in their fermentation of tryptophane in the absence of sugar as a source of carbon, carry the fermentation through both stages to produce first 3-indoleacetic acid and then indol. When indol-positive bacteria are grown in artificial media with tryptophane, and sugar as the preferred source of carbon, only the first stage in the breakdown of tryptophane is carried out and 3-indoleacetic acid is the final product. Indol is produced from the fermentation of tryptophane by indol-positive bacteria only when the sugar supply is exhausted. 3-indoleacetic acid is but a stage in the process of indol formation; it cannot serve as proindol. Indol-negative bacteria form 3-indoleacetic acid from tryptophane, but the process never, of course, culminates in the production of indol. The second stage of the process, the conversion of 3-indoleacetic acid to indol, does not occur. Sugar merely stimulates the growth of indol-negative bacteria by providing additional nutrient and does not effect the manner in which tryptophane is broken down; in the presence of sugar as the carbon source the process still goes through only the first stage to produce 3-indoleacetic acid.

Nielsen (1930b) has shown that rhizopin[1] accelerates the growth velocity of yeast. Boysen Jensen (1931) found that bacteria from human saliva, *E. coli*, and other bacteria produced growth substances which, when applied in an agar block to one side of an oat coleoptile, caused it to bend negatively. An abundant formation of growth substance was brought about only with growth of bacteria upon solid media. It is probable that this growth substance, like rhizopin, is heteroauxin (Went and Thimann, 1937).

Boysen Jensen (1931a)[2] mentions the possibility that the "growth regulator" produced by microorganisms may accelerate their growth velocity. If this is true the presence of such a substance in media would be advantageous since growth of bacteria in such media occurs at a faster rate. However, Went and Thimann (1937) point out that, so far as is known, the 3-indoleacetic acid produced by fungi has no effect upon their growth. No mention is made of the possibility that this hormone may have an influence upon the growth rate of bacteria.

[1] Thimann (1935a) has demonstrated rhizopin to be 3-indoleacetic acid.

[2] Footnote, page 245.

MATERIALS AND METHODS

Cultures of *Escherichia coli* were grown in a synthetic liquid medium in order to avoid the effects of organic growth factors other than heteroauxin. The constituents of this medium[3] were:

K_2HPO_4	0.50 gram
KH_2PO_4	0.50 gram
$MgSO_4 \cdot 7H_2O$	0.20 gram
NaCl	0.01 gram
$FeSO_4 \cdot 7H_2O$	0.01 gram
l-tryptophane[4]	0.10 gram
Glucose	10.0 grams
Distilled water	1 liter

3-indoleacetic acid[4] was added to this medium in concentrations ranging from one to ten parts per ten million parts of medium. The pH of the culture fluid was 6.4.

Inoculations were made from 24-hour-old cultures of *E. coli*, that had been grown in nutrient broth at 37°C., into tubes that contained 5 cc. of the synthetic liquid medium. These source cultures contained approximately 2,700,000 cells per mm^3. The volume of the loop used in inoculations was approximately 0.8 mm^3 and contained approximately 2,160,000 bacterial cells.

Counts were made of 48-hour-old cultures in the synthetic medium that had been incubated at 37°C. The apparatus used was a Petroff-Hauser bacterial counting chamber with an oil immersion microscope. Cultures were grown and counted in triplicate for each concentration of heteroauxin from one to ten parts per ten million of culture fluid. For controls, counts were made of cultures grown under the same conditions and in the same medium containing no heteroauxin.

EXPERIMENTAL RESULTS

The experimental results are given in table 1.

DISCUSSION AND CONCLUSION

The counts of *E. coli* given show that concentrations of heteroauxin between one and ten parts per ten million parts of culture fluid give approximately the same quantitative stimulus to cell division. The number of cells was more than doubled in the medium containing the hormone as compared with the number of cells in control tubes that contained no hormone. These figures indicate that growth, as measured by cell division, is not proportional to the concentration of heteroauxin within these limits. Therefore, the major function of heteroauxin is apparently that of a stimulus to cell division in *E. coli*. In the experiments outlined, the bacterial cells divided faster in the presence of artificially added heteroauxin than they did without it.

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[3] Adapted from Snell, Tatum and Peterson, Jour. Bact. **33**, 207, 1937.

[4] Obtained from Eastman Kodak Company.

TABLE 1

Experimental results

CONC. P.P. 10^7 OF HETEROAUXIN	COUNT 1, CELLS PER MM^3.	COUNT 2, CELLS PER MM^3.	COUNT 3, CELLS PER MM^3.	AVERAGE
0				92,000*
1	190,000	226,000	228,000	214,000
2	222,000	226,000	202,000	216,000
3	190,000	226,000	214,000	210,000
4	200,000	226,000	266,000	230,000
5	182,000	208,000	266,000	218,000
6	186,000	216,000	238,000	214,000
7	156,000	222,000	240,000	206,000
8	236,000	194,000	218,000	216,000
9	204,000	202,000	228,000	212,000
10	220,000	202,000	212,000	212,000

* Average of 17 counts.

As to the effect of heteroauxin upon the size of the bacterial cell and upon the morphology of cells in old cultures, no studies were made. It is quite possible, however, that heteroauxin may influence the size of the bacterial cell and also that it may have a rôle in the induction of pleomorphism. It is left for future studies to determine this.

Hopkins and Cole (1903) have shown that *E. coli* produces 3-indoleacetic acid from tryptophane. The results of this investigation show that there is a pronounced stimulus brought about in the growth of the organism by the hormone. The relation of heteroauxin to the growth of this organism appears therefore to be that of a self-activating system; heteroauxin is produced in the normal growth processes of these bacterial cells by their oxidative deamination of tryptophane (Went and Thimann, 1937). The heteroauxin so formed apparently stimulates the cells to further growth and division which produces more cells and more heteroauxin.

Boysen Jensen (1932) established that *Aspergillus niger* was able to produce growth substances by the breakdown of the following amino acids besides l-tryptophane: l-histidine, l-leucine, d,l-phenylalanine, d-lysine and l-tyrosine. When agar blocks containing these growth substances were applied to one side of an oat coleoptile, negative curvatures resulted. The possibility must be considered that bacteria, in fermenting amino acids other than tryptophane, also produce growth substances that should cause bending of the oat coleoptile as well as an acceleration of their own growth velocity.

This mechanism of growth stimulus for a bacterium may be compared to that which was postulated by Söding (1937) for the effect of auxin of higher plants upon cambial growth. He presented evidence that the growth substance, which comes from the bud, is conducted downward for a short distance in the stem only in the cambium. In the cambial cells exposed to the auxin, there is a stimulation of growth and cell division which produces more auxin. The auxin so formed is again conducted downward a short distance in the cambium where the cycle of initiation of growth and the production of additional auxin comes about: the wave of growth that goes down the stem is thus explained.

The stimulation of the bacterial cell by heteroauxin to produce more heteroauxin and normal growth by cell division may also be compared to one theory of virus action. As postulated by Schultz (1930) and Rivers (1932), a virus-tainted cell is stimulated to the production of more of the same agent which induced the original disease process and the abnormal growth of the cells characteristic of the disease.

SUMMARY

Escherichia coli was grown in a synthetic culture fluid which contained from one to ten parts of heteroauxin per ten million

parts of fluid. Counts were made in triplicate of cultures of each of these concentrations of the hormone. Regardless of the concentration of the hormone used in the medium the same stimulus of cell division was obtained. The cell division of the organism was more than doubled in the medium containing the hormone as compared with that in control tubes containing no hormone.

Comparisons are drawn with Söding's theory of the action of growth substances in the cambium of higher plants and with a modern theory of the action of viruses.

I wish to make acknowledgments for favors done in the course of this work to Dr. H. L. Chance, under whose direction the work was carried out, to Mrs. Leena J. MacArthur for the loan of equipment from the School of Pharmacy, to Dr. K. V. Thimann of Harvard University for valuable advice and criticism and to Dr. G. L. Cross for suggestions concerning the manuscript.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MISSOURI VALLEY BRANCH

OMAHA, NEB., MAY 14, 1938

THE EFFECT OF SULFANILAMIDE ON THE STREPTOCOCCI IN THE UDDER OF MASTITIS COWS. *Henry Bauer and M. F. Gunderson*, Department of Pathology and Bacteriology, College of Medicine, University of Nebraska, Omaha, Nebraska.

Eight cows infected with chronic mastitis were treated with doses of sulfanilamide ranging from one-half to sixteen ounces. These doses were fed over a period of twelve hours to twenty-two days. The amount of drug excreted was determined at hourly or daily intervals. Fuller's method for the estimation of sulfanilamide in blood was modified for the estimation of the drug in milk. Curves revealing the sulfanilamide concentration in blood and milk were obtained. The streptococcal flora of the treated cows was followed for several months by means of incubated milk samples of which Breed smears were made, blood agar plates, brom-thymol-blue and Hotis tests.

Sulfanilamide exerted a transient effect on the streptococci in the udder of the infected cows. As long as the drug could be detected in the milk, the streptococcal count remained low and in some instances disappeared; however, as soon as the udder was cleared of the drug, the streptococci reappeared in numbers as great as before treatment was begun.

One of the eight cows was infected with staphylococci; the drug showed no effect on these organisms.

MESENCHYMAL REACTIONS AND BODY DEFENSE. *Wilhelm Voigt*, University of Nebraska, College of Medicine, Omaha.

The work of Wilhelm Ehrich (Ehrich and Wohlrab, Ehrich and Voigt in Beitr. pathol. Anat., Vol. **92**, 2) has been extended. There is a relationship of the condition of immunity and the occurrence of cellular reactions of the vascular connective-tissue-apparatus. Intensive reactions of the intima and adventitia of the small vessels of lung, liver and spleen were noted upon the intravenous injection of a heat-killed culture of *Staphylococcus aureus*. The sizes of the germinal centers in lymphnodes vary directly with the agglutinin titers.

FACTORS FACILITATING THE CULTIVATION OF NON-SPORULATING ANAEROBIC BACILLI FROM THE INTESTINAL TRACT. *Margaret Bedell and Keith H. Lewis*, Department of Bacteriology, University of Nebraska, Lincoln, Nebraska.

A study of the growth-promoting properties of a variety of gases, mineral salts, carbohydrates, proteins, infusions, and extracts has led to the preparation of a relatively simple, clear, heat-stable medium of the following composition:

	per cent
Tryptone	2.0
Bacto beef extract	1.0
Glucose	0.5
Cysteine hydrochloride	0.05
Di-sodium phosphate (hydrate)	0.4

Dissolve all ingredients in distilled water, adjust to pH 7.4, and sterilize in the autoclave for 20 minutes at 120°C.

While this medium supplies all nutrients essential for rapid development of the non-sporulating anaerobic bacilli, it has been noted also, (1) that the exclusion of carbon dioxide from the atmosphere completely inhibits growth, and (2) that the addition of liver infusion, yeast extract, and tomato juice stimulate more profuse growth.

Studies on Members of the Escherichia-Aerobacter Group Isolated from Kansas Ice Cream.

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Presumptive tests for presence of *Escherichia-Aerobacter* group were made by adding measured amounts of melted ice-cream to lactose peptone bile 2 per cent broth. Of the 51 samples examined, 40 yielded positive presumptive tests, 37 of which were confirmable. On each of the 24 samples in the second series, 16 of which gave confirmable tests for organisms of the *Escherichia-Aerobacter* group, phosphatase tests for pasteurization efficiency were made. Four samples were positive to both tests indicating that possibly the organisms were present because of inefficient pasteurization. Twelve samples were phosphatase negative but still contained *Escherichia-Aerobacter* organisms indicating contamination after pasteurization or the presence of resistant strains. Each colony from positive presumptive tests was isolated and studied. Of 86 cultures obtained, 54 were *Aerobacter*, 26 were *Escherichia*, and 6 were *Citrobacter*. All were killed by 30 minutes exposure at 142°F. using sterile skim milk as a suspension medium but 12 *Escherichia* survived the exposure when sterile ice-cream was used.

Indications are that organisms of the *Escherichia-Aerobacter* group are frequently present because of contamination following pasteurization, although inefficient pasteurization may be the reason for their presence under some circumstances. The use of higher temperatures than are used for the pasteurization of milk for the processing of ice cream mix is again indicated by the resistance of a number of *Escherichia*-strains to exposure to 143.5°F. for 30 minutes when suspended in ice cream mix.

Sulfanilamide Levels in the Blood of Rabbits with and Without Fever Treatment.

Murray D. Lewis, M.D., and Millard F. Gunderson, Ph.D., Department of Bacteriology, University of Nebraska, College of Medicine, Omaha.

The recent use in man of combined sulfanilamide and fever therapy, particularly for gonorrhea, suggested the necessity for observing animals subjected to this combined treatment.

Fifteen rabbits were fed 1 gram sulfanilamide per kilo and placed in a cabinet modeled after the Kettering Hypertherm. The temperature of these animals was kept at 107–108°F. for 9–10 hours. Ten survived the treatment. The blood sulfanilamide reached higher levels than in the five that died, suggesting that the cause of death was not due to sulfanilamide over-dosage but rather hyperpyrexia. The maximum blood sugar level was 36 mgm. per cent and this was not reached until the sixth hour contrasting with the 57 mgm. per cent at the third hour in the unheated controls. The slower rise in blood sulfanilamide in these 10 animals to a lower maximum may possibly be attributed to less rapid absorption from the gastro-intestinal tract in the presence of high fever. The fact that 10 out of 15 animals treated in this fashion survive without permanent ill-effect 9 or 10 hours of fever at 107–108°F. suggests that the feeding of large doses of sulfanilamide increases the hazard of artificial fever therapy not at all, or only slightly.

As controls, rabbits were fed 1 gram per kilo of sulfanilamide but were not put in the fever cabinet. These animals all showed signs of shock with cyanosis, coldness, and fall in rectal temperature from 101–102°F. to 99–100°F. The figures for blood sulfanilamide levels in these animals is given above.

THE INFLUENCE OF ADSORBED MAGNESIUM ON LEGUME BACTERIA. *T. M. McCalla*, Kansas State College, Manhattan.

A neutral colloidal clay system containing Mg, PO_4, K, and Ca proved superior to a similar system in which Ba was substituted for Mg as a medium for maintaining normal strains of these organisms.

DETOXIFICATION OF TYPE C BOTULINIUM TOXIN BY PROTEINS. *M. F. Gunderson*, Department of Pathology and Bacteriology, University of Nebraska, College of Medicine, Omaha, Nebraska.

Doses of three cubic centimeters of Type C botulinum toxin, 0.001 cc. of which was fatal by injection, were fed to pigeons. Death ensued in less than twelve to twenty-four hours.

Similar amounts of toxin, mixed with 0.5 cc. fresh egg-albumen, and fed to pigeons failed to kill until seven to fifteen days elapsed, and, in instances, death did not result.

The binding can be dissociated so that the toxin will quickly exert its effect in spite of the presence of protein.

FIBRINOLYTIC ACTIVITY OF STREPTOCOCCI OF HUMAN AND ANIMAL ORIGIN. *John D. Lemar and Millard F. Gunderson*, Department of Pathology and Bacteriology, University of Nebraska College of Medicine, Omaha, Nebraska.

Forty streptococci of human origin and seventy streptococci of animal origin were tested for their fibrinolytic activity upon fibrin obtained from a number of sources. These included human, horse, bovine, swine, sheep, hog, dog, guinea pig, rabbit, pigeon and duck fibrins.

The human streptococci were obtained, with a few exceptions, from superficial infections, as were those of animal origin. The veterinary sources included horse, cow, swine, pig, sheep, guinea pig, rabbit, fox, and chicken.

The streptococci of human origin were active against all fibrins, though this was not marked in the case of fibrins obtained from swine, sheep, dog, horse, and guinea pig. The greatest activity was against human fibrin.

Some activity was displayed by the animal streptococci against all fibrins. This was most marked against human fibrin. Horse and pigeon fibrin were only slightly susceptible.

In both groups of organisms, prolonged incubation (96 hours) enhanced the fibrinolytic powers of most of the organisms. In the few exceptions fibrinolytic power was not changed, decreased, or lost entirely.

In this study there seemed to be no correlation between species-pathogenicity and specific fibrinolytic power. Fibrin of human origin was more susceptible to lysis by both groups of organisms than was any other.

CONSTITUTIONAL CHANGES IN RABBITS SUBJECTED TO COMBINED FEVER AND SULFANILAMIDE THERAPY. *Lucile Loseke and M. F. Gunderson*, University of Nebraska, College of Medicine, Omaha, Nebraska.

Normal healthy rabbits were kept for 5–6 hours in a heated cabinet with controlled humidity, their temperatures reaching 108°F. Blood taken immediately after the animals were removed from the fever cabinet showed a moderate increase in hemoglobin and red count, slight decrease in white count, and relative increase in pseudo-eosinophiles and decrease in lymphocytes. Non-protein-nitrogen was increased one-third, blood sugar levels, doubled, and CO_2 combining power decreased. Rabbits treated with sulphanilamide and also untreated rabbits were given fever treatments during which time red, white, differential, and blood sugar values were obtained, first at 15 minute intervals; later at hourly periods. In all cases an initial drop occurred in red and white counts during first part of fever period. The red count gradually increased after the initial drop so that it usually exceeded the normal count at the end of 75 hours of fever. The white count increased after an initial drop and the granulocytes increased at the expense of the lymphocytes. Blood sugar commenced to be increased almost at once after the exposure in the heated cabinet.

Studies were also made on rabbits treated with small daily doses of sulfanilamide for several weeks and others treated with sulfanilamide daily and fever weekly. These animals all maintained their weight as long as they lived. Blood sugar and non-protein-nitrogen showed no significant change from week to week. The carbon dioxide combining power decreased slightly. The difference between total hemoglobin and hemoglobin as determined by oxygen capacity method varied from 0.1 to 3.0 grams per 100 cc. of blood.

THE METHOD OF ELECTRICAL CONDUCTIVITY IN STUDIES ON BACTERIAL METABOLISM[1]

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Received for publication April 14, 1938

The electrical conductivity of solutions depends upon the concentrations and the mobilities of charged particles of varying sizes. A culture medium in which bacteria are living and dying is a heterogeneous system, the composition and complexity of which are constantly changing. Substances in true and in colloidal solution are being chemically disintegrated and synthesized. The initial and final compositions may be well-known, but the intermediate reactions and the mechanisms involved in many cases are not understood. Several difficult problems are therefore presented in attempting to evaluate conductivity measurements in relation to bacterial metabolism. Parsons and Sturges (1926a, b) have reported a definite correlation between conductivity and the amount of ammonia and amino nitrogen produced by putrefactive anaerobes. This is the only direct relationship between conductivity and some product of metabolism which has been established so far. Others have shown, however, (Sierakowski and Lęczyeka, 1933 and papers quoted therein) that there is always some relationship between change in electrical conductivity and metabolic processes even though the direct cause for the alteration in electrical field strength may be obscure. Further information on such correlations has been sought. Experiments have been done, therefore, with two different aerobic bacteria which produce ammonia, and one which produces lactic acid. It is believed that the data presented show the value of conductance measurements in describing, identifying, and interpreting the intermediary metabolism of these organisms.

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[1] Read before the New York City Branch of the Society of American Bacteriologists, May 11, 1937.

[2] The writers are indebted to Messrs. Darwin Vexler, Arthur E. Orloff, and Oscar Beder for assistance in determinations and in many other ways.

DESCRIPTION OF ORGANISMS

One of the ammonia-forming organisms was *Pseudomonas fluorescens*, recently isolated from milk. It conformed in all respects to the type description in Bergey's Manual of Determinative Bacteriology.

The other ammonia-forming organism was a lipolytic bacterium isolated from rancid cream by Anderson (Anderson and Hardenbergh, 1932). Since this is undoubtedly a new organism the name *Achromobacter lipidis*, N. Sp., is proposed because of its pronounced fat-splitting ability. The organism is a gram-negative, non-spore-forming rod, about 0.4 by 1.6 microns, occuring singly and in pairs. It appears to be non-motile or very sluggishly motile. Flagella have not been detected. Agar colonies are about four or five millimeters in diameter after forty-eight hours of incubation at 25–28°C., after which they slowly increase in size for several days. These colonies are circular with an entire edge, smooth, glossy, moist, and finely granular. With age they become coarsely granular and develop radial striations which result in a more or less undulate margin. Young colonies are white and beautifully opalescent. They do not darken with age. The agar slant growth is abundant, glistening and echinulate. Agar stab cultures produce an abundant surface growth with a delicate subsurface growth, diminishing rapidly with depth. In broth the upper portion of the tube first becomes turbid and a slight surface film develops; later the turbidity spreads throughout the entire tube and a characteristic slimy white precipitate accumulates. An alkaline reaction due to ammonia is produced in broth. An abundant moist white growth occurs on potato slants. Gelatin is not liquified by freshly isolated cultures, but cultures which have been grown on meat extract agar for some months may cause a slight liquification of gelatin in ten days. No indol is produced in Dunham's solution. Nitrates are readily reduced to nitrites. The organism is incapable of fermenting glycerol, glucose, galactose, fructose, sucrose, lactose, maltose, raffinose, inulin, starch, dulcitol, and mannitol. Litmus milk is slowly reduced and then becomes alkaline, commencing at the surface. The alkaline reaction is due, in part at least, to ammonia production. Colonies on blood agar plates are not larger than on plain agar and no hemolysis of blood cells or change in hemoglobin occurs. The outstanding characteristic of this organism is its marked lipolytic action, even at low temperatures. Freshly isolated cultures grow best at 28–29°C., fail to grow above 34–35°C., and show a growth after several days at 2–4°C.

The lactic-acid forming organism studied was *Lactobacillus odontolyticus* obtained from the culture collection of E. R. Squibb and Company through the courtesy of Dr. G. F. Leonard.

MATERIALS AND METHODS

The conductivity cells made from short-necked flasks varied in capacity from 250 cc. to 1000 cc. The platinized electrodes were inserted through openings made in the shoulder of the flasks. These electrodes varied in size and in separation according to the requirements of the experiment. Another opening was made near the top of the flasks so that samples could be drawn from time to time for chemical analysis. The slide wire in a Leeds and Northrop student potentiometer was used as the Wheatstone bridge. The instrument was shielded, and grounded through a Wagner Ground. The 1000-cycle current was supplied by a calibrated audio-oscillator. The bridge was balanced by obtaining a minimum sound through telephones. The resistance was accurately measured to 0.1 ohm and the capacitance to 0.001 microfarad. The cell constants were checked before and after each run using N/50 KCl solution as a reference solution.

Each medium was sterilized in a conductivity cell and the whole was brought to the temperature of the incubator before inoculation. The temperature of the incubator was kept optimum for each organism.

pH determinations on milk inoculated with *L. odontolyticus* were made by the quinhydrone electrode. The pH of one-per-cent peptone solutions inoculated with the lipolytic organism was determined colorimetrically. The pH of milk inoculated with *P. fluorescens* was determined by the glass electrode developed by MacInnes (MacInnes and Belcher, 1933). Van Slyke's micro-amino-nitrogen apparatus (Van Slyke, 1912) was used to determine amino nitrogen. Care was taken to remove all the ammonia from the sample before analyzing for amino nitrogen since it has been shown that results obtained without the removal of ammonia are 18 to 50 per cent too high, depending upon the temperature and the size of sample (Parsons and Sturges, 1926c). Ammonia was removed by Folin's aeration method (Folin and MacCullum, 1912) as well as by Van Slyke's vacuum distillation method (Van Slyke, 1911). The ammonia was analyzed colorimetrically after Nesslerization. The CO_2 in the media was determined manometrically using Van Slyke's apparatus (Van Slyke, 1917). Bacterial populations were counted by the standard agar-plate method. There was no evidence of contamination.

EXPERIMENTS ON PROTEOLYSIS

The data obtained from the analyses of skim milk inoculated with *Pseudomonas fluorescens* are listed in table 1. After an initial adjustment, increase in specific conductivity was found to be proportional to the amount of ammonia formed. This fact is illustrated in figure 1 where the line drawn through the experimental points is described by the following equation:—

$$(NH_3) = 6.08 \times 10^3 \, \Delta \, C + 2 \qquad (1)$$

where (NH_3) refers to the concentration (millimols per liter) of ammonia formed and Δ C to the increase in specific conductivity of the medium. A similar proportionality was reported by Parsons and Sturges (1926a, b) in their work on conductivity and ammonia production by certain putrefactive anerobes. Their proportionality constant was, however, somewhat greater, which means that an increase in conductivity represented the synthesis of more ammonia in their media inoculated with putrefactive anerobes than in the skim milk medium inoculated with *P. fluorescens.* They also found this proportionality between ammonia and increase in specific conductivity to apply from the origin. The data presented here show a change in mechanism which prevents the line from going through the origin and results in the addition of the constant 2.

TABLE 1

Data obtained from the analyses of skim milk inoculated with Pseudomonas fluorescens

Temperature 28 ±0.5°C.

TIME	SPECIFIC CONDUCTIVITY × 10^3	AMMONIA	AMINO NITROGEN	CARBON DIOXIDE	$\frac{NH_3^*}{CO_2}$	pH
hours		*millimols per liter*	*millimols per liter*	*millimols per liter*		
0	6.58	0.0	13.6	0 42	0.00	6.16
23	6.66	0.1765	14.4	1.05	0.28	6.20
47	6.67	1.945	19.9	2.185	1.10	6.24
70	6.85	4.23	33.5			
94	7.11	5.71	32.3	5.38	1.15	6.54
119	7.38	7.12				6.60
141	7.61	8.65	33.7	6.94	1.32	6.62
170	8.00	10.10	51.3	7.49	1.42	6.75
192	8.37	12.35	51.1	9.15	1.41	7.05
216	8.70	13.05	62.3			
240	9.18	18.23	64.0			
265	9.54	17.65	64.2	12.44	1.46	7.06
336	10.32	27.20	73.2	19.19	1.45	7.02
384	10.98	31.20	80.5	21.65	1.46	
432	11.66	34.10	105.1	28.80	1.20	7.27
456	12.10	35.90	95.4	32.88	1.10	
504	12.48	36.40	114.8	39.65	0.92	7.30
552	12.95	42.0	109.0	44.50	0.95	7.40
574	13.12	41.4	114.1	47.50	0.88	7.37
600	13.30	41.6	132.0	54.70	0.76	7.42

* See text for method of calculation.

The data obtained from the analyses of a peptone medium inoculated with *Achromobacter lipidis* are listed in table 2. The medium was a one-per-cent solution of Difco peptone in distilled water. Although the quantity of ammonia formed in the peptone medium was less than that formed in skim milk inoculated with *P. fluorescens,* there is still a direct proportionality between the increase in specific conductivity and millimols of ammonia. This direct proportionality, which holds from the origin, is illustrated in figure 2. It will be noted that there is a definite break in the mechanism of the reaction after the specific conductivity has been increased to approximately 0.64 x 10^{-3} mhos.

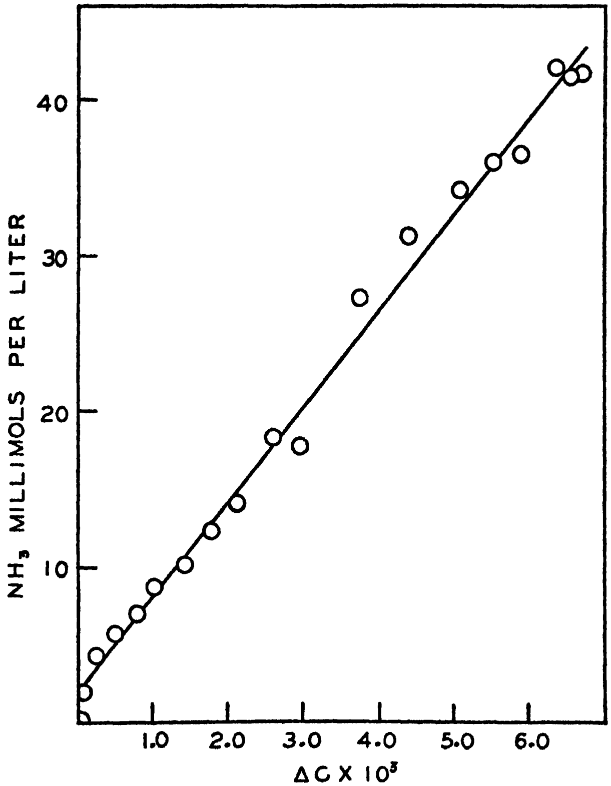

FIG. 1. Increase in specific conductivity ($\Delta C \times 10^3$) of skim milk inoculated with *Pseudomonas fluorescens* plotted against the concentration of ammonia (NH_3) expressed in millimols per liter.

This sudden increase in ammonia has been noted qualitatively in other samples of peptone media inoculated with this organism. Such a change in the mechanism of reaction would be interesting to study in detail, especially if it should prove to be characteristic of this organism.

The equation which describes the first straight line drawn in figure 2 is

$$(NH_3) = 10.86 \times 10^3 \Delta C \qquad (2)$$

TABLE 2

Data obtained from the analyses of 1 per cent peptone medium inoculated with Achromobacter lipidis, n. sp.

Temperature 28 ±0.3°C.

TIME	SPECIFIC CONDUCTIVITY $\times 10^3$	AMMONIA	CARBON DIOXIDE	$\frac{NH_3}{CO_2}$[1]	BACTERIA	pH
hours		*millimols per liter*	*millimols per liter*		*millions per cc.*	
0	0.9144	0	0.415	0	0.2567	7.15
10	0.9222				1.8367	
18	0.9414		1.04		9.333	
24	0.9566				118.7	7.20
30	0.9802		1.66			
32					134.3	
42	1.0363	1.46	2.48	0.70	141.7	
56	1.0787	1.77	3.286	0.61	241.7	
68	1.1168		3.73		263.0	
76	1.1537				235.3	7.42
80	1.1815	3.48	4.94	0.76	279.0	
92	1.2128	3.23	4 80	0.73	378.0	7.55
104	1.2416	3.17	4.70	0.74		
116	1.2718	4.40			392.33	7.60
125	1.3239				446.0	7.65
130	1.3461	4.69	5.58	0.90		7.75
134	1.3585	5.00				
140	1.3930	5.16			395.7	7.88
143					365.7	
160	1.5345	5.55	6.00	0.95		7.92
168					363.7	
201	1.6404	12.50	6.24	2.14		8.12
209	1.6291	12.00				
258	1.6369	12.91	6.35	2.17		
264					330.0	
307	1.6739	15.26	5.68	2.90		8.28

[1] See text for method of calculation.

where (NH_3) represents the millimols of ammonia per liter and ΔC the increase in specific conductivity. Although less ammonia is produced in the experiment described for this organism

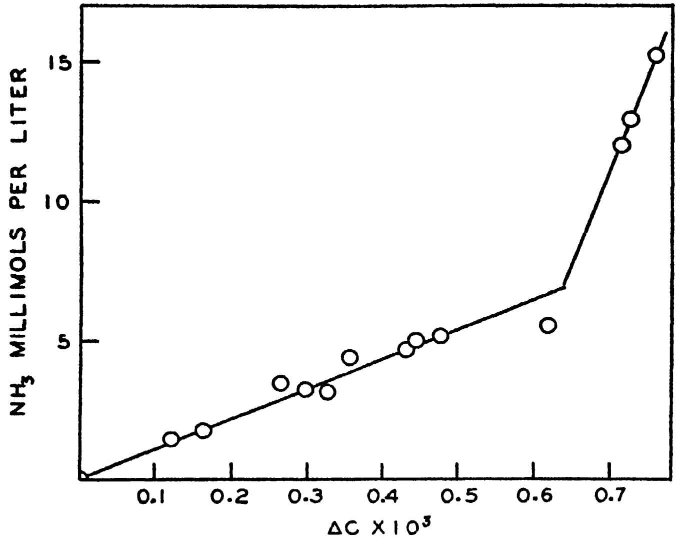

FIG. 2. Increase in specific conductivity ($\Delta C \times 10^3$) of a one per cent peptone medium inoculated with *Achromobacter lipidis, n. sp.*, plotted against the concentration of ammonia (NH_3) expressed in millimols per liter.

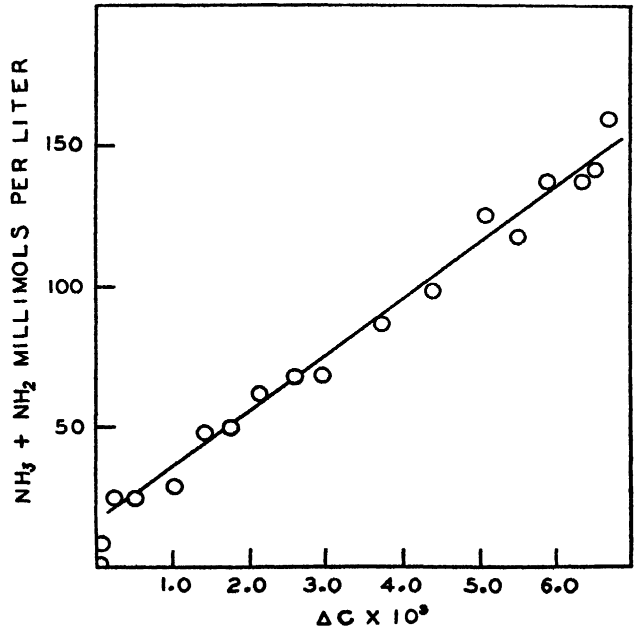

FIG. 3. Increase in specific conductivity ($\Delta C \times 10^3$) of skim milk inoculated with *Pseudomonas fluorescens* plotted against the concentration of ammonia plus the increase in concentration of amino nitrogen ($NH_3 + NH_2$) expressed in millimols per liter.

than that described for *P. fluorescens*, the proportionality constant is higher.

Parsons and Sturges (1926a, b) also found that the increase in conductivity was proportional to the increase in the nitrogen calculated from a formol titration; in other words to the increase in ammonia plus the increase in amino nitrogen. The data obtained on *P. fluorescens* shown in figure 3 illustrate such a proportionality. The equation for the line is

$$(NH_3) + (NH_2) = 20.0 \times 10^3 \, \Delta \, C + 16 \qquad (3)$$

where (NH_3) refers to the millimols of ammonia per liter and (NH_2) represents the increase in millimols of amino nitrogen per liter. An initial change in mechanism is apparent here, as well as in the curve for ammonia, since the line in figure 3 does not pass through the origin.

DISCUSSION OF RESULTS ON PROTEOLYSIS

Free carboxyl and free amino groups appear in approximately equal amounts as a protein is hydrolyzed. Changes in conductivity resulting from enzymatic hydrolysis of a protein must be correlated in some way with the appearance of these polar groups in solution. Northrop (1919–1920) showed that while the liberation of carboxyl groups through hydrolysis of egg albumin with pepsin would increase conductivity, the liberation of free amino groups would decrease conductivity because some of the free acid would be bound by these basic groups. Thus, increase in conductivity would not necessarily parallel increase in hydrolysis of the protein. Baernstein (1928) in a similar study demonstrated, however, that there were two regions of pH where conductivity increased in direct proportion to the increase in amino nitrogen produced through enzymatic hydrolysis of albumin. One region, pH 1.38 or less, is quite acid while the other region, pH 7.36, is on the basic side of the isoelectric point of many amino acids and polypeptides. He points out that his results are consistent with the "Zwitterionen" theory of Bjerrum (1923). Thus, in solutions sufficiently acid, the conductivity will be influenced by the concentration of cations formed.

The problem of studying proteolysis by living bacteria is not as simple, however, as one which involves the enzyme alone. Bacteria, for example, not only hydrolyze proteins but also deaminize some of the amino groups which are liberated. Ammonia, as well as amino nitrogen, appears in solution. A certain unknown quantity of this nitrogen derived from protein is utilized by the bacteria in the synthesis of protein and other nitrogenous derivatives. The sum, then, of the ammonia plus the amino nitrogen (equation 3) in the medium will be less than the amount of nitrogen actually liberated through the hydrolysis of the proteins. Waksman and Starkey (1932) have pointed out that nitrogen may also be lost from the culture medium as ammonia if there is a marked increase in alkalinity. There was, however, no loss in total nitrogen in the skim milk culture of *P. fluorescens*, since the total nitrogen of the cultures remained the same as the controls. The quantities, therefore, of ammonia and amino nitrogen must be correlated in some way with each other, with the degree of hydrolysis, and with specific conductivity.

According to equations 1 and 3 it is possible to deduce that there is a direct proportionality between the increase in millimols of amino nitrogen and the increase in specific conductivity. The pH range is near that which can be predicted from Baernstein's work to give a direct proportionality between amino nitrogen and increase in conductivity. Incidentally the narrowness of this change in pH makes negligible any effect of an increase in (OH^-) on conductivity. Parsons and Sturges (1926) conclude that the increase in conductivity of their cultures of putrefactive anerobes can be accounted for entirely by the increase in concentration of ammonium salts. They believe that compounds containing amino nitrogen, such as the amino acids, polypeptides, etc., would not contribute much to conductivity.

It appears from our analysis, however, that either ammonia or amino nitrogen is directly proportional to specific conductivity. Further, it is possible to deduce from the data presented here on *P. fluorescens* that the change in concentration of ammonia is directly proportional to the change in amino nitrogen, or

$$d(NH_3)/d(NH_2) = K = 0.437 \qquad (4)$$

It follows that the rate of ammonia formation is 43.7 per cent of the rate of amino-nitrogen formation or,

$$d(NH_3)/dt = 0.437\, d(NH_2)/dt \qquad (5)$$

The production and activity of the enzymes involved in the catalysis of hydrolytic and synthetic processes which yield ammonia and amino nitrogen must be closely correlated. Equation 5 can be interpreted to mean that constant proportions of amino acids are formed and deaminized, as well as utilized in nitrogen metabolism, by these organisms. This does not mean, however, that ammonia and amino nitrogen production by microorganisms will always bear this close relationship. For example, Waksman and Lomanitz (1925) have indicated that some microorganisms tend to accumulate amino acids in the early stages of proteolysis. Later, the amino acids are broken down and ammonia nitrogen predominates. Possibly some such change as the latter explains the break in the curve shown in figure 2. Other possibilities suggest themselves but they are all correlated with the growth and development of the organism itself. Thus, a complete understanding of proteolysis involves a knowledge of the intermediary metabolism of each organism.

Bacterial metabolism refers to the coordinated chemical reactions which take place in the complex heterogeneous system of bacteria and medium. The mechanism of coordination is largely unknown although some kind of a relationship between variables such as ammonia, CO_2, (H^+), numbers of bacteria, specific conductivity, etc., must exist. There is, for example, some significance to the ammonia/carbon-dioxide ratios. These ratios are listed in tables 1 and 2 and they are calculated by dividing the millimols of ammonia produced per liter, by the increase in millimols of carbon dioxide in the medium at any time. In the early hours of growth of *P. fluorescens*, carbon dioxide production is greater than ammonia production; i.e., the ammonia/carbon-dioxide ratio is less than unity. As the growth in population proceeds, this ratio gradually increases in value until it has reached approximately 1.4 at 160 hours which is almost the maximum. The maximum ratio shows that for every mol of CO_2 held in the medium, almost one and one-half mols of ammonia are produced. The increase in the ammonia/carbon-dioxide ratio with time can be interpreted to mean either that the organisms are utilizing sources of carbon richer and richer in nitrogen or that less and less nitrogen is required by the population of bacteria for their synthetic needs. As the population matures, the mechanism of metabolism becomes more constant and a maximum ratio is established. The decline in this ratio which begins at approximately 400 hours would mean that the organisms are more and more using carbon compounds poor in nitrogen as a source of energy. Compounds, for example, which could accumulate from the metabolism of amino acids during the growth of the population might be utilized at this time.

The ammonia/carbon-dioxide ratios listed in Table 2 are essentially constant from 42 hours until a slight rise occurs at 130 hours. This can be interpreted to mean a constant mechanism of metabolism over this period. The slight increase in the ratio at 130 hours leads to a greater rise to a new value over 2.0, slightly above the ratio calculated for ammonium carbonate. This is a change in mechanism of reaction which is in accord with the change illustrated in figure 2. It is possible that the large increase in ammonia is some function of the change in concentration of enzymes secreted by the bacteria which are involved in converting amino groups into ammonia.

These speculations emphasize the fact that if the mechanisms of coordination between these variables were known, then the measurement of a single variable such as specific conductivity would permit an integration of the whole process of metabolism.

Further studies need to be made to establish these mechanisms of coordination.

EXPERIMENTS WITH L. ODONTOLYTICUS

The data obtained in the study of the souring of skim milk through the metabolism of *L. odontolyticus* are listed in table 3 and shown graphically in figure 4. Conductivity cell no. 1 was not shaken during the experiment. Cell no. 2 was shaken before each determination. The result was that after the curd formed it remained firm and unbroken in cell no. 1, but was broken and settled around the electrodes in no. 2. Conductivity changed in both flasks, as illustrated in figure 4, at approximately the same rate up to the point where the curd began to form. Where the curd was not broken, in cell no. 1, the conductivity

TABLE 3

Data obtained from the analyses of skim milk inoculated with Lactobacillus odontolyticus

Temperature 37.65 ±0.15°C. Cell #1 contained 250 cc. of the medium and it was not shaken during the period of analysis. Cell #2 contained 750 cc. of the medium and it was shaken before every determination. Samples for pH determinations were withdrawn from Cell #2.

TIME	SPECIFIC CONDUCTIVITY $\times 10^3$, CELL 1	SPECIFIC CONDUCTIVITY $\times 10^3$, CELL 2	pH
hours			
0	7.705	7.614	6.47
2	7.774	7.77	6.45
4	7.863	7.809	6.39
6	7.969	7.916	6.27
8	8.086	8.02	6.23
10	8.297	8.274	6.10
12	8.450	8.525	5.95
14	8.737	8.687	5.71
16	8.978	8.88	5.57
18	9.177	9.08	5.53
20	9.367	9.335	5.39
22	9.564	9.706	5.22
24	9.775	9.797	5.05
26	10.024	9.948	4.96
28	10.18	10.105	4.80
30	10.305	10.175	4.68
32	10.415	10.205	4.63
34	10.545	10.215	4.52
36	10.695	10.235	4.40
38	10.728	10.225	4.31
40	10.736	10.225	4.20
42	10.761	10.225	4.10
44	10.815	10.225	4.11
46	10.838	10.235	4.05
48	10.854	10.25	3.93
55	10.94		
57	10.92		

continued to rise. No further change in conductivity took place in cell no. 2 after the curd was broken and allowed to settle around the electrodes. These facts suggest that in the former case, where the curd remained unbroken, a colloidal gel was formed so that minute particles of casein did not hinder the passage of a current of electricity between the two electrodes in cell no. 1 as much as in cell no. 2.

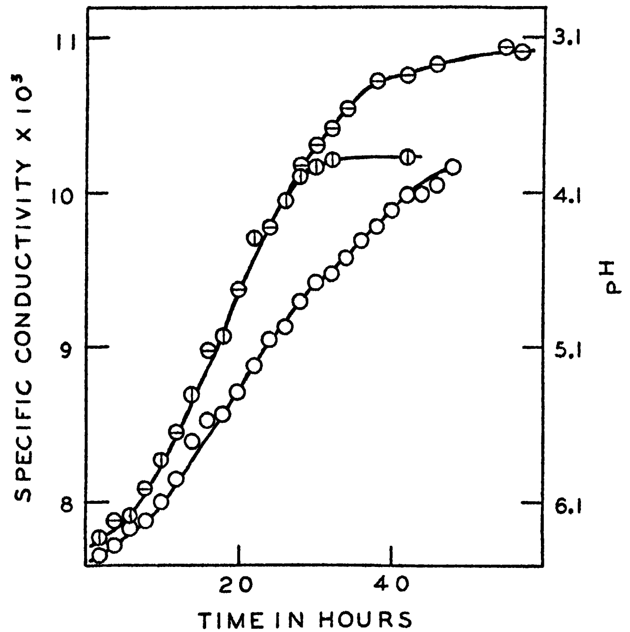

Fig. 4. Time in hours plotted against specific conductivity and pH of skim milk inoculated with *Lactobacillus odontolyticus.* ⊖ represent determinations of specific conductivity in conductivity cell no. 1 which was not disturbed throughout the experiment. ⦶ represent determinations of specific conductivity in conductivity cell no. 2 which was shaken before each determination. The open circles ○ represent pH determinations made on samples taken from cell no. 2.

Conductivity changes in this system before the curd is formed are primarily due to the increase in the concentration of the hydrogen ion. Other factors, however, play a rôle in this increase in electrical field strength of the medium. Probably the most important of these other factors is the acid-binding capacity of the casein. Thus, as the lactic acid is formed through the decomposition of lactose, a certain number of hydrogen ions will be removed from solution by combining with the protein. The protein will gradually lose its charge until at the isoelectric point the casein separates from solution. The net result is an approximate linear relationship between specific conductivity and pH up to the isoelectric point.

The value of conductivity measurements in the investigation of the metabolism of lactic-acid-forming bacteria is obvious. Further study should yield information concerning the physical chemistry of the reaction in milk as well as establish the rate of acid formation as it is correlated with other variables that are also a result of the metabolism of these organisms. Conductivity measurements could be used also to advantage in a comparative study of the rate of acid formation between different lactic acid forming organisms. A preliminary analysis, for example, has shown no difference in the rate of formation of lactic acid by *Lactobacillus bulgaricus* and *Lactobacillus odontolyticus* using change in conductivity as a measure of acid formation.

SUMMARY

An ammonia-forming lipolytic organism isolated from cream is named *Achromobacter lipidis*, n. sp., and is described. Specific conductivity is directly proportional to the production of ammonia by *Pseudomonas fluorescens* grown in skim milk and by *Achromobacter lipidis*, n. sp., grown on one-per-cent peptone medium. The processes which result in the formation of ammonia and amino nitrogen in skim milk inoculated with *Pseudomonas fluorescens* are interdependent; the rate of ammonia formation being 43.7 per cent of the rate of amino-nitrogen formation. The ratio between increase in ammonia and increase in carbon dioxide is an aid in the interpretation of the mechanism of metabolism. Measurements made on skim milk inoculated with *Lactobacillus odontolyticus* demonstrate that specific conductivity is a linear function of decreasing pH until the isoelectric point of the casein is reached. A constant specific conductivity is approached rapidly if the curd precipitates around the electrodes while the approach is slower if a colloidal gell is formed.

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NITROGEN AVAILABILITY AS AN AID IN THE DIFFERENTIATION OF BACTERIA IN THE COLI-AEROGENES GROUP

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Received for publication April 30, 1938

The colon group of bacteria may be characterized as nonsporing Gram-negative bacilli which ferment lactose, producing acid and gas, and which can grow aerobically. Although many bacteriologists recognize the genera *Escherichia* and *Aerobacter*, the genus *Citrobacter* has not been generally accepted.

In 1923, Koser showed that strains of the genus *Aerobacter* utilized citric acid as a sole source of carbon (citrate positive), whereas those of the genus *Escherichia* did not (citrate negative). In 1924, Koser found that 23 of 25 coliform strains [methyl-red (+), Voges-Proskauer (−)] isolated from unpolluted soil were citrate (+). Due to their source he referred to them as "soil forms." The possible sanitary significance of this finding was immediately realized. The correlation of habitat with morphology and biochemical characteristics was, and still is, the chief hope for an improved criterion of water pollution.

Bardsley (1934) summarized previous work on this point: "Most workers in tropical countries where pollution is very heavy find it practical to distinguish between the different types of coliform bacilli, and only those organisms which conform to the true *B. coli* group [methyl-red (+), citrate (−)], are regarded as excretal contamination.... In temperate climates the position is not so well defined."

Fermentation of cellobiose and alpha-methyl-glucoside, production of trimethylene glycol from glycerol, and even Werkman and Gillen's (1932) description of the genus *Citrobacter* as comprising those citrate (+) coli-like strains which produce "Acetoin . . . rarely from glucose and then only in traces" have been found inadequate for differentiation of the "intermediates" from strains of the genera *Escherichia* and *Aerobacter*. A clearer characterization of the "intermediate" group is necessary.

The physiological characterization of the colon group has been stressed, in the past, from the standpoint of carbohydrate chemistry though some work with several purine compounds has been reported. Plenge (1903) and Schittenhelm and Schröter (1903, 1904) reported that "*B. coli*" attacked nucleic acid. Koser (1918) showed that *Aerobacter*, but not *Escherichia* strains, utilized uric acid as a sole nitrogen source. He obtained similar results with hypoxanthine hydrochloride, although here the *Aerobacter* strains grew less luxuriantly. Chenn and Rettger (1920) confirmed Koser's work on uric acid and found that xanthine gave similar results with typical *Escherichia coli* strains. But of 20 coli-like strains from soil, 10 of which were uric acid (+) and 10 uric acid (–), all were xanthine (–).

Nucleic acid and its degradation products were selected for this study because (1) of the known fact that uric acid is an available nitrogen source for *Aerobacter* but not for *Escherichia* strains; and, (2) of the possibility of correlating the findings with work in biochemistry.

According to Levene (1920), yeast nucleic acid is composed of the following radicals: 4 phosphate, 4 carbohydrate, 2 purine (adenine and guanine), and 2 pyrimidine (uracil and cytosine). Uric acid is the main end-product of purine digestion in man and anthropoid apes; allantoin is the chief end-product in most other animals. Allantoin is converted, *in vitro*, to hydantoin and urea, and further to glycine, carbon dioxide and ammonia.

The following investigation of the availability of yeast nucleic acid and some of its degradation products, (adenine sulphate, xanthine, uracil, uric acid, allantoin, hydantoin, and urea) was made with a view to development of differential media for the colon group. All but urea were Eastman products.

The basal medium consisted of: 0.5 per cent NaCl; 0.02 per cent $MgSO_4$; 0.004 per cent brom-thymol blue; and approximately 2 per cent by volume of 1.0 molar phosphate buffer (pH 7.1). In some media it was necessary to employ a more alkaline phosphate buffer solution in order to adjust the reaction to approximately pH 7.1. To determine the availability of a compound as a source of nitrogen, 0.2 per cent of glucose and 0.05 per cent of the test compound were added to the basal medium; to ascertain the availability of a compound as a sole source of carbon, 0.2 per cent of monoammonium phosphate was supplied as an available nitrogen source, and to determine the availability of a compound as a sole source of both carbon and nitrogen, 0.5 per cent of the test compound was added to the basal medium described above.

All glassware was acid washed and rinsed with sodium bicarbonate solution followed by distilled water. All chemicals were chemically pure. The constituents were dissolved in conductivity water at a temperature below boiling. Since the effect of autoclaving these compounds was unknown, all media were sterilized by filtration through Chamberland L3 candles. Inoculums consisted of a one-millimeter loop of a 24-hour broth culture. Incubation was for four to five days at 30°C. (temperature of the medium).

The bacteria employed in the nitrogen availability studies included: 106 *Escherichia*; 39 *Aerobacter cloacae*; 31 *Aerobacter aerogenes* (indol positive); 41 *Aerobacter aerogenes* (indol negative); 138 "intermediate" strains (75 of which were contributed by workers outside this laboratory); and 6 strains which were received as "intermediates" but which gave questionable Voges-Proskauer reactions. Included among the "intermediate" strains was a transfer of the original strain of *Citrobacter freundii*, the type species of the genus *Citrobacter*.

Frozen eggs constituted the source of 90 per cent of *Escherichia*, 33 per cent of "intermediate," 77 per cent of *Aerobacter*, and one of the 6 Voges-Proskauer questionable strains. The source of isolation was unobtainable for 48 per cent of the "intermediate" and 3 of the Voges-Proskauer questionable strains. The rest of the cultures were secured from fowl or human feces, surface water, swimming pool water, etc. The date of isolation, or of arrival at this laboratory, varied from 1929 to 1936. The "intermediate" and questionable Voges-Proskauer strains were purified by at least three platings on eosin methylene-blue agar. The *Escherichia* and *Aerobacter* strains, from the Iowa State College bacteriology laboratory collection, were checked, as to lactose, eosine methylene-blue agar, Voges-Proskauer, and citrate reactions.

TABLE 1

Summary of differential characteristics of the coli-aerogenes group

	ESCHERICHIA	INTERMEDIATE	AEROBACTER	VP (?)	CITROBACTER FREUNDII
	Number of strains				
	106	138	111	6	1
	Per cent of positive reactions				
Yeast nucleic acid........	0	0	96	100	–
Uric acid.................	0	0	100	100	–
Allantoin.................	0	0	100	100	–
Hydantoin.................	0	0	87	100	–
Uracil....................	80	2	100	100	–
Urea......................	2	92	100	100	+
VP........................	0	0	100		–
Citric acid...............	0	100	100	100	+
MR........................	100	95	0	100	+
Cellobiose................	2	95*	99	100	Acid, no gas
H_2S†......................	1	83†	0	0	+
Indol.....................	98	6	23	17	–

* Of the 131 "intermediate" strains which attacked cellobiose, 97 produced acid but no gas.

† Vaughn and Levine (1936) medium.

Availability of a compound as a nitrogen source was indicated by production of acid from the glucose in the medium. In all experiments evidence of growth as indicated by increased turbidity, was also recorded.

In table 1 are summarized the results for tests in which the entire collection of cultures was employed.

Adenine was tested with only a few strains (10 *Escherichia*, 10 *Aerobacter* and 8 "intermediates") and xanthine was observed with 10 *Escherichia*, 10 *Aerobacter* and 50 intermediate strains. The nitrogen of xanthine and adenine was utilized by all the coli strains tested. Since no differential action was evidenced, other cultures were not tested, and these compounds are therefore not listed in table 1.

It was observed that the *Aerobacter* and questionable Voges-Proskauer strains utilized the nitrogen of all the compounds listed; whereas *Escherichia* strains utilized the nitrogen of the pyrimidine uracil only, while the "intermediates" utilized only the nitrogen of urea.

None of the strains of *Aerobacter*, after having acidified the medium, reversed the reaction in nucleic acid, xanthine (questionable reversal for a few strains), or hydantoin media, although they did so in media containing other nitrogen sources. A few *Aerobacter* strains showed beginning reversal of reaction in the uracil medium on the third day, but complete reversion to the original alkalinity did not occur in any case by the fifth day. The "intermediate" and *Aerobacter* strains showed such rapid reversion in the urea medium that observations for short incubation periods ($<$ 24 hours) were necessary in order to detect acid production.

In media containing adenine, xanthine and urea increase in turbidity was well correlated with development of acidity as shown by intensity of color change of indicator. The *Aerobacter* in nucleic acid, and the *Escherichia* strains in uracil, however, produced a distinct acidity without markedly increasing turbidity.

In media employing uric acid or allantoin as sole sources of nitrogen there was perfect agreement between the two indices (turbidity and acidity) for utilization of a test compound when strains of *Aerobacter aerogenes* were used. Of the 39 strains of *Aerobacter cloacae*, however, all produced acid while only 31 per cent (12 strains) showed vigorous growth as evidenced by marked turbidity. In the hydantoin medium, 60 per cent of the *Aerobacter cloacae* strains produced acid after 3 days at 30°C. but in no case was the turbidity greatly increased. Without further chemical study the significance of the differences observed with the two criteria as indicators of nitrogen utilization is conjectural. Acid production from an available sugar in the presence of a test compound as a sole nitrogen source appeared to have the greater practical value for differentiation of members of the colon group.

Vigor of growth as indicated by increased turbidity was employed as the criterion of availability of carbon, and of both carbon and nitrogen. Observations with 10 *Escherichia,* 8 "intermediates," 5 *A. aerogenes,* and 5 *A. cloacae* strains indicated that nucleic acid, uracil, uric acid, allantoin, hydantoin, and urea were utilized as carbon and as both carbon and nitrogen sources by *A. aerogenes,* but not by the other strains. Adenine sulphate served as an available source of carbon, but not of both carbon and nitrogen, for the *A. aerogenes* strains; it was not utilized by the other strains.

The effect of moderate autoclaving (15 lbs. for 12 minutes, and the medium cooled immediately) on availability of the test compounds as nitrogen sources was observed with nucleic acid, uracil, allantoin, hydantoin, and urea. Inoculation with the above 28 test strains showed that, as nitrogen sources, neither nucleic acid nor uracil was appreciably affected by autoclaving while allantoin, hydantoin, and urea were distinctly modified. Prior to autoclaving, the allantoin and hydantoin compounds had not served as nitrogen sources for either "intermediate" or *Escherichia* strains; after autoclaving they were available as nitrogen sources, for the "intermediate" but not for the *Escherichia* strains. The filtered urea-nitrogen medium supported a vigorous growth and acid production of "intermediates," but *Escherichia* strains did not grow. Autoclaving rendered the medium suitable for *Escherichia* strains. Further work might develop the autoclaved allantoin and hydantoin media as useful in differentiating the "intermediate" from the *Escherichia* strains.

In an experiment employing five *Escherichia* strains it was observed that their differentiation from *Aerobacter* in the allantoin medium was associated with a small inoculum. Heavy seeding from an agar slant presumably introduces some factor which permits abundant multiplication of *Escherichia* strains and therefore invalidates the differential value of the medium.

This necessity for employing a small inoculum is known to hold for several accepted differential tests e.g., Koser's uric acid medium.

DISCUSSION

Since methods for qualitative and quantitative determination of many of the intermediate products of decomposition of nucleic acid are available from the field of physiological chemistry, a study of bacterial fermentation of these compounds should be greatly facilitated. As the *Aerobacter* and questionable Voges-Proskauer strains are able to utilize all of the nitrogen compounds studied it is suggested that the disintegration of nucleic acid by these strains may be analogous to that occurring in the animal digestive tract, and that a study of the bacterial fermentation of these compounds offers a possible method of investigating the course of their digestion in the animal intestinal tract.

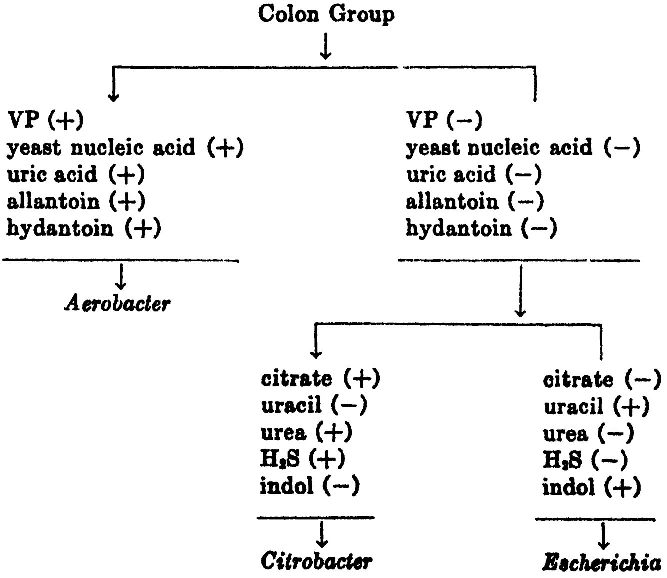
Lucke (1931) presented evidence that a great part of the uric acid of the gastric juice and bile is destroyed in the alimentary canal. He found that this destruction commenced in the upper small intestine. Since it is known that strains of *Aerobacter* predominate in that region, the present report makes it logical to assume that nucleic acid or any of its degradation products in the intestine could be readily disintegrated by bacteria as well as by the digestive enzymes of the host.

It would be interesting to know the point in the molecule at which these nitrogen compounds are attacked. The *Aerobacter* strains utilized all compounds tested and therefore offer no clue. That the *Escherichia* and "intermediate" strains could not attack the imidazole ring or that this ring is inhibitory is suggested by their failure to attack uric acid, allantoin, and hydantoin. However, the *Escherichia* strains attacked uracil, and the "intermediates" attacked urea. The evidence for the suggestion offered is slight; other imidazole ring compounds as nitrogen sources must be studied before the suggestion could be accepted as anything other than an hypothesis.

One may then inquire why xanthine and adenine sulphate were utilized as nitrogen sources. At present, there appears to be no logical explanation of the action on xanthine. As to the utilization of the nitrogen of adenine sulphate, it is to be borne in mind that various radicals may greatly affect the availability of a compound to bacteria. For instance, glucose and alpha-methylglucoside are not attacked with the same ease by the colon group, and methyl urea and thiourea are not equally available nitrogen sources to the same bacteria (de Jong). It may be that the sulphate radical renders the adenine more easily attacked.

Perusal of table 1 shows that, on the basis of utilization of degradation products of yeast nucleic acid in unautoclaved media as sole nitrogen sources, the colon group may be separated into three divisions: one which utilized all of the compounds tested, a second which utilized only uracil, and a third which utilized only urea. The questionable Voges-Proskauer strains were wholly like those of the genus *Aerobacter* with respect to their utilization of these nitrogen compounds and were therefore allocated to that genus.

From table 1 a dichotomous chart may be formed:



These data may be considered as strengthening the evidence for allocating the "intermediate" strains to a separate genus *Citrobacter*. About 90 per cent of the "intermediates" gave reactions identical with those of a transfer of the original strain *Citrobacter freundii*, the type species of the genus *Citrobacter*. It is therefore suggested that, if *Citrobacter* is retained, those strains of the coli-aerogenes group having the following characteristics be allocated to that genus:

Gram-negative short rods; do not produce spores; ferment lactose with acid and gas; do not produce acetylmethylcarbinol (VP negative); utilize citric acid as a sole source of carbon; generally produce H_2S in appropriate media; utilize urea as a sole source of nitrogen but not yeast nucleic acid, uracil, uric acid, allantoin or hydantoin; and ferment cellobiose with acid, but generally no gas production.

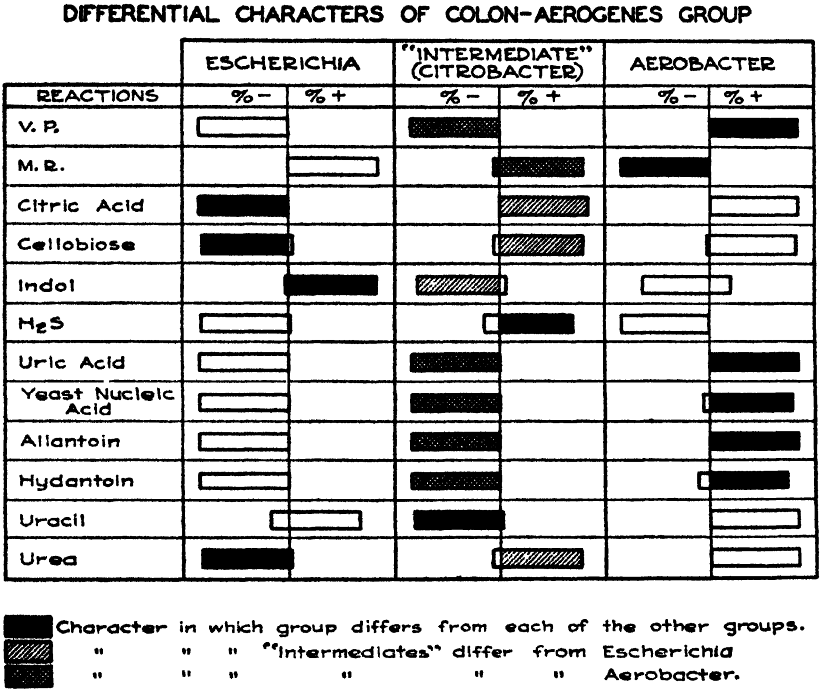


FIG. 1

The reactions of the genera *Escherichia*, *Aerobacter* and *Citrobacter* with respect to the twelve characters studied are shown graphically in figure one. It will be noted that the genus *Es-*

cherichia differs from the other two genera in that it does not utilize citric acid or cellobiose as sole sources of carbon, nor urea as a sole source of nitrogen. The genus *Aerobacter* differs from the other two genera in that it is Voges-Proskauer positive, methyl-red negative, and utilizes uric acid, nucleic acid, allantoin and hydantoin as sole sources of nitrogen. The "intermediate" group (*Citrobacter*) differs from both of the other genera in that it produces hydrogen sulphide from appropriate media and, particularly, in that it does not utilize uracil as a source of nitrogen.

The genus *Citrobacter*, as defined above, differs from *Escherichia* in that it is capable of utilizing urea but not uracil as a sole source of nitrogen, is indol-negative, and can utilize citric acid and cellobiose as sole sources of carbon. It differs from the genus *Aerobacter* in that it is Voges-Proskauer negative, methyl-red positive and cannot utilize uric acid, nucleic acid, allantoin, hydantoin, or uracil as sole sources of nitrogen.

SUMMARY

Studies on the availability of nucleic acid and certain of its degradation products for members of the colon group of bacteria revealed generic differences.

The availability of nucleic acid and its degradation products when correlated with the Voges-Proskauer reaction, citrate utilization, hydrogen sulphide, and indol production, lends support to the allocation of the "intermediate" strains to a separate genus *Citrobacter*.

The colorimetric test based on the production of acid from glucose in a synthetic medium proved to be a convenient and practical method for ascertaining utilization of nucleic acid and its degradation products as sole nitrogen sources.

The nitrogen-availability studies offered a means of classifying strains giving questionable Voges-Proskauer reactions. In the present study six such strains investigated were allocated to the genus *Aerobacter*.

Limited observations indicated that of the colon strains investigated only *Aerobacter aerogenes* utilized nucleic acid, uric

acid, uracil, allantoin, hydantoin, and urea as sole sources of carbon or of both carbon and nitrogen.

Exploratory runs indicated that neither nucleic acid nor uracil was affected as a nitrogen source by moderate autoclaving, whereas allantoin, hydantoin, and urea were distinctly altered. In the present study all media were sterilized by filtration.

A more detailed chemical study is suggested. Investigation of the nitrogen availability of other pyrimidine compounds should prove fruitful.

The genus *Citrobacter* as defined in this paper differs from *Escherichia* in that the former (1) utilizes urea but not uracil as a sole source of nitrogen, (2) utilizes citric acid and cellobiose as sole carbon sources, and (3) produces H_2S but not indol. It differs from *Aerobacter* in that it (1) gives a positive methyl-red test, (2) forms H_2S in proteose peptone ferric citrate agar, (3) does not produce acetylmethylcarbinol (Voges-Proskauer negative), and (4) does not utilize nucleic acid, uric acid, allantoin, hydantoin, or uracil as nitrogen sources.

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SUGAR ALCOHOLS

XVII. THE UTILIZATION OF SORBITOL, STYRACITOL, SORBOSE, PINITOL, PRIMULITOL AND HYDROXYPYRUVIC ALDEHYDE BY VARIOUS MICROÖRGANISMS

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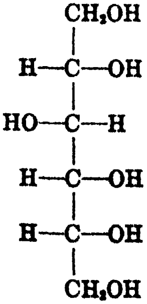
Received for publication May 25, 1938

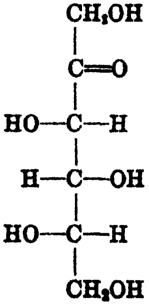
In former communications, the authors (1935, 1936, 1937) presented studies on the utilization of several sugar alcohols and their anhydrides by various microörganisms. It was observed that the removal of a molecule of water from a sugar alcohol with the formation of its anhydride, in most instances, prevented its utilization by many organisms of the colon-aerogenes group. Since the anhydrides of dulcitol and mannitol are not utilized, it was thought of interest to study the anhydride of sorbitol which is a corresponding sugar alcohol.

The additional compounds, sorbose, pinitol, primulitol and hydroxypyruvic aldehyde were available and hence were included in these studies.

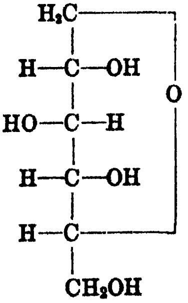
The following formulas show the structural relationship of the substances employed:



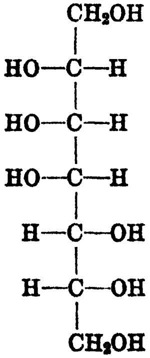
d-Sorbitol



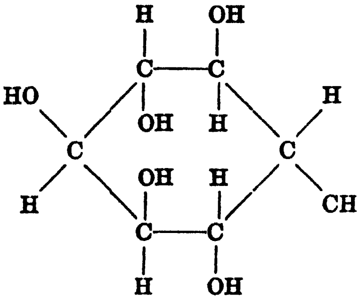
1-Sorbose



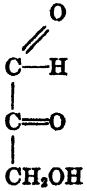
Styracitol
1-5 Anhydro sorbitol



Primulitol
β-d-Mannoheptitol



Pinitol
Mono methyl *d*-inositol



Hydroxypyruvic
Aldehyde

COMPOUNDS STUDIED

Sorbitol is a non-reducing, sweet-tasting, crystalline sugar alcohol prepared from d-glucose by reduction with sodium amalgam. Pfanstiehl's C. P. sorbitol was used in these studies. l-sorbose is the ketose isomeric with fructose. It was discovered in 1852 in Mountain-ash berries along with sorbitol. Sorbose is not believed to occur naturally in the plant but to be formed by the oxidizing organism, *Bacterium xylenium,* acting upon sorbitol. Commercially, sorbose is prepared by the action of *Bacterium xylenium* upon sorbitol. Merck's C. P. sorbose was employed in this investigation.

Styracitol, the 1–5 anhydride of sorbitol, corresponds to polygalitol.[1] It is a stable, non-reducing, sweet-tasting, crystalline compound. It was synthesized from glucose in this laboratory by the method of Zervas (1930) which involves the reduction and hydrolysis of tetraacetyloxylglucal.

Primulitol (β-d-mannoheptitol) is a seven-carbon-atom sugar alcohol unlike any compound so far studied in this series. It was extracted from the plant root, *Primula offinicalis* by the method of Bougault and Allard (1902). It is non-reducing.

Pinitol is a seven-carbon-atom cyclo hexitol with a methyl group attached to one carbon atom. The compound was extracted from *Pinus libertiana* by the method of Sherrard and

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[1] Recently Freudenberg and Rogers have presented evidence to show that styracitol is 1-5 anhydromannitol and polygalitol 1-5 anhydrosorbitol. J. A. C. S. 59: 1602 (1937).

Kurth (1928). The compound melted uncorrected between 183 and 185°C.

Hydroxypyruvic aldehyde is entirely different from the compounds so far studied as far as its chemical reactivity is concerned. It is very reactive, reducing Fehling's solution in the cold. The compound was prepared by the method of Evans *et al.* (1938) and the form used was the trimer of the alcoholate.

METHOD

In this study 25 strains of *Escherichia coli*; 14 propylene-glycol-positive, and 11 propylene-glycol negative (Dozois *et al.* 1937) were used. Of the 14 strains of "intermediates" studied, 10 were adonitol and inositol negative while 4 fermented these sugars. Seven strains of *Aerobacter aerogenes*, 4 of *Eberthella typhosa* and 11 of *Salmonella* were studied. The characteristics of each organism were determined in detail.

Fermentation tests were carried out in 1-per cent carbohydrate broth culture media, prepared by adding the sterile carbohydrate to sugar-free nutrient broth of a pH of 7.0. Bromthymol blue or bromcresol purple was added to serve as an indicator. Incubation was at 37.5°C. for 14 days and observations for the formation of acid and gas were made daily. Each strain was carried through at least 4 serial passages to determine its continued ability to utilize the carbohydrate in question.

RESULTS

With the exception of 1 strain of *Aerobacter aerogenes* all organisms studied fermented sorbitol. All of the propylene-glycol-positive and 2 of the propylene-glycol-negative *Escherichia coli*, and all of the "intermediates" fermented sorbose. The *Eberthella typhosa* were unable to utilize it, while the members of the *Salmonella* group formed acid but no gas with this sugar.

Ten of the propylene-glycol-positive *E. coli* fermented styracitol. Three propylene-glycol-negative strains produced only acid from styracitol while 6 other members of this group completely fermented it. This anhydride was utilized by all of the

Aerobacter aerogenes studied. The reaction of the adonitol-inositol-negative "intermediates" varied; 2 organisms fermented

TABLE 1

Utilization of sorbitol and anhydrides

ORGANISMS STUDIED	SORBITOL			SORBOSE			STYRACITOL		
	A	A*	Θ	A	A*	Θ	A	A*	Θ
Escherichia coli (25 strains):									
Propylene-glycol-positive		14			14			10	4
Propylene-glycol-negative		11			2	9	3	6	2
Aerobacter aerogenes (7 strains)		6	1	3	2	2		7	
Intermediate (14 strains):									
Adonitol-Inositol-negative		10			10		1	2	1
Adonitol-Inositol-positive		4			4			6	4
Eberthella typhosa (4 strains)		4				4			4
Salmonella (11 strains):									
paratyphi		5		5			5		
schottmuelleri		6		6				6	

A = Acid formation.
A* = Acid and gas formation.
Θ = No fermentation.

TABLE 2

ORGANISMS STUDIED	PINITOL			PRIMULITOL			HYDROXYPYRUVIC ALDEHYDE		
	A	A*	Θ	A	A*	Θ	A	A*	Θ
Escherichia coli (25 strains):									
Propylene-glycol-positive		8	6			14			14
Propylene-glycol-negative			11			11	3	2	6
Aerobacter aerogenes (7 strains)		2	5			7	3		4
Intermediate (14 strains):									
Adonitol-Inositol-positive			4		4		1	2	1
Adonitol-Inositol-negative			10	1	5	4	6		4
Eberthella typhosa (4 strains)			4			4	4		
Salmonella: (11 strains)									
paratyphi			5			5	5		
schottmuelleri		6			6				6

A = Acid formation.
A* = Acid and gas formation.
Θ = No fermentation.

it, 1 produced only acid and 1 failed to utilize it. Six adonitol-inositol-positive "intermediates" gave positive results. The *Eberthella typhosa* gave negative results with styracitol, the *Salmonella* *paratyphi* produced acid but no gas while the *Salmonella schottmuelleri* utilized it.

Pinitol was fermented by 8 strains of propylene-glycol-positive *E. coli*, 2 strains of *A. aerogenes*, and 6 strains of *Salmonella schottmuelleri*.

All of the *E. coli*, *A. aerogenes*, *E. typhosa* and *S. paratyphi* failed to utilize primulitol. The adonitol-inositol-negative "intermediates" as well as 6 strains of *Salmonella schottmuelleri* formed both acid and gas from primulitol.

The fermentation results with hydroxypyruvic aldehyde were varied. The propylene-glycol-positive *Escherichia coli* failed to utilize this aldehyde; of the propylene-glycol-negative group, 2 completely fermented it and 3 produced only acid. Three of the *Aerobacter aerogenes* strains produced acid but no gas from this aldehyde. Of the 4 strains of adonitol-inositol-positive "intermediates" 1 produced acid and 2 completely fermented hydroxypyruvic aldehyde. Six adonitol-inositol-negative "intermediates", 4 strains of *Eberthella typhosa* and 5 *Salmonella paratyphi* also produced acid but no gas from this aldehyde.

CONCLUSIONS

1. The removal of one molecule of water from sorbitol which results in the formation of styracitol alters its utilization by many bacteria of the colon-aerogenes group.
2. Pinitol, primulitol and hydroxypyruvic aldehyde fermentation studies may lead to further methods of distinguishing *Salmonella paratyphi* from *Salmonella schottmuelleri*.
3. Since none of the "intermediates" studied fermented pinitol and some of the *Escherichia* group were able to utilize it, this sugar alcohol may be of value in the identification of the group.
4. The fermentation reactions of the colon-aerogenes group on pinitol, primulitol and hydroxypyruvic aldehyde are so varied that more complete studies are necessary to determine their taxonomic value.

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BACTERIAL OXIDATION-REDUCTION STUDIES

I. DIFFERENTIATION OF SPECIES OF THE SPORE-FORMING ANAEROBES BY POTENTIOMETRIC TECHNIQUE[1]

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Received for publication May 31, 1938

The value of research in the field of bacterial oxidations and reductions is obvious when the results which have accumulated on this subject in recent years are reviewed. The significance of such phenomena has been established on a sound basis only recently. Although the methods applied to the quantitative study of the oxidation-reduction potentials of biological systems are still subject to certain errors, it can hardly be denied that the serious pursuit of the many "unknown quantities" existent in this field may lead to valuable practical developments in medicine and the industries, and, perhaps more important, to a more complete comprehension of fundamental aspects of biological processes.

The observations to be reported here cover one aspect of a rather intensive study of the electrode potentials of anaerobic cultures of various bacteria. Evidence will be presented that cultures of certain species of the spore-forming anaerobic group exhibit rather marked and constant differences in O/R potential, with respect to both maximum reduction intensity and trend of potential drift. Moreover, it appears that not only species differences exist, but that strains within a species exhibit a certain degree of individuality.

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[1] This paper represents a part of the dissertation presented to the Graduate School of Yale University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The cost of the original equipment used in this work was met by a grant from the Fluid Research Fund of the Yale University School of Medicine.

Potter (1911) appears to have been the first to report changes in the electromotive character of a medium in which bacteria were growing. He attributed the potential difference observed between an uninoculated (sterile) and an inoculated medium, both of the same original composition, to the bacterial decomposition which resulted in the latter. He associated this effect with the similar phenomenon which occurs in sugar solutions subjected to the action of enzymes or to acid hydrolysis.

That certain groups of bacteria may be characterized by definite reduction potentials exhibited in their cultures was first suggested by the work of Gillespie (1920). Having observed the markedly different final reduction potentials which develop in cultures of strict aerobes, as contrasted with those of facultative anaerobes or of mixed cultures presumably containing strict anaerobes, he hazarded the opinion that these differences may apply generally to the two distinct groups of bacteria, aerobes and anaerobes.

Cannan, Cohen and Clark (1926) offered an explanation for the potential differences observed in cultures of different bacteria which was based on differences in metabolic activity. This theory is supported by the results of subsequent investigations in this field.

Hewitt (1930, 1931, and 1933) succeeded in differentiating certain types of bacterial species by means of potentiometric technique. The characteristic potentials of certain of the species studied were attributed to the ability or inability of the organism to form peroxide.

In 1934 Sagen, Riker and Baldwin correlated the oxidation-reduction potentials of cultures of two plant pathogens, *Phytomonas tumefaciens* and *Phytomonas rhizogenes*, and *Achromobacter radiobacter*, with certain physiological characteristics which these organisms exhibit. The two *Phytomonas* species showed a tendency to establish potentials at different levels of intensity. This appears to be the first report indicating differentiation of closely related species by this method. It should be observed, however, that the differences in H-ion concentration recorded by them appear to have been sufficiently large to have influenced the results decidedly.[2]

The observations of Tuttle and Huddleson (1934) on differentiation of *Brucella* species seem to be associated with bacteriostasis and are, therefore, probably not significant as a demonstration of the differential value of final reduction potentials.

It was shown quite recently (1935 and 1936) by Burrows and Jordan that species of the *Salmonella* group exhibit very distinct differences in final reduction potential.

The results of experiments conducted by us (1936) do not agree with those of Plotz and Geloso (1930) with respect to the negative potential limits established in cultures of various species of *Clostridium*. These investigators reported that all of the spore-forming anaerobic species studied by them reached and maintained very nearly the same degree of reducing intensity. Their observations were made on cultures held *in vacuo*. We have found appreciable differences in the final reduction potentials established by several species in this genus. Dissimilarity in technique may, perhaps, explain the lack of agreement.

APPARATUS AND TECHNIQUE

Considerable time and effort were devoted to the development of the apparatus and methods to a point where they could be applied successfully to the study of electrode potentials of bacterial cultures under strictly controlled conditions. With the exception of the units of electrical equipment, the apparatus was constructed in this laboratory, using materials assembled from various sources. The final outfit proved to be admirably adapted for the study of a wide variety of problems in the field of bacterial oxidation-reduction.[3]

The gas-purifying and distributing device was modeled after the one described by Knight (1930 and 1931), but with certain minor alterations and additions. All connections between the different elements of the entire gas system were made with heavy-walled pressure tubing, which was lubricated with "Cello-Seal" and liberally covered with paraffin. The connections were practically "glass to glass." At points where a limited flexibility was desired, the rubber tubing was covered with gold size and then well coated with "Cello-Seal."

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[2] Evidence is to be presented in a subsequent publication that changes in H-ion concentration which occur in bacterial cultures affect the reduction potentials to a very appreciable extent.

[3] A full description of this apparatus and the technique employed in this series of investigations will be found in the Ph.D. dissertation of the senior author in the Sterling Library of Yale University.

The electrode-culture vessels used in these studies were essentially the same as the type described by Clark and Cohen (1923, 1 and 2), Knight (1930 and 1931) and others. Large hard-glass test tubes about 1½" in diameter and 6½" in length were employed for this purpose. Each vessel was fitted with a No. 8 rubber stopper which supported duplicate electrodes, a KCl-agar bridge, gas inlet and exit tubes, and a sampling tube. A battery of eight vessels (Hewitt, 1933) was used in each experiment. The vessels were incubated in an ordinary bacteriological incubator at 37°C.

Anaerobic conditions were established by flushing the vessels thoroughly with tank nitrogen which was purified by passage over heated reduced copper. Except in the earlier work, the passage of purified nitrogen was maintained throughout the experiment, to insure a positive pressure within the system and thus minimize the possibility of leakage of air into the culture vessel. Leakage was encountered very infrequently. The continued passage of gas had the added advantage of preventing the accumulation of metabolic gases in the cultures and vessels. The influence of certain of the gases arising from this source upon the potentials will be discussed later.

In certain instances it was found desirable to mix limited quantities of CO_2 with the purified nitrogen before distribution to the vessels, because of the stimulatory effect of this gas upon the development of some types of bacteria. In some of the investigations it was necessary to establish and maintain definite potential levels in the culture media. This was done (Knight, 1930 and 1931) by passing definite mixtures of purified and impure nitrogen through the cultures.

The vessels were constructed to facilitate sampling, which was accomplished by means of a Pasteur pipette inserted into the vessel through a short section of glass tubing of small bore; the tubing passed through the vessel stopper and was fitted with a tight rubber cap. During the sampling process the cap was removed, allowing the gas passing through the vessel to escape while the specimen was being taken. Thus, no air was admitted into the vessel to disturb equilibrium. This same technique was occasionally used for introducing inoculum or various reagents into a de-aerated medium or culture.

Duplicate electrodes of platinum wire bent into spiral form were used in each vessel, except in certain instances when one of the oxidation-reduction electrodes was replaced by a specially prepared glass electrode, in order that pH changes might be observed more frequently and with less effort than could be done by sampling. The electrodes were prepared in such a way that not less than 2 cm. of 22 gauge platinum wire was exposed to the medium or culture. They were cleaned as recommended by Hewitt (1933), and were kept in distilled water when not in use. When these precautions are neglected the electrodes do not behave satisfactorily. Duplicate electrodes frequently agreed exactly. They rarely varied more than 2 or 3 mv.

In more recent studies of the sporulating anaerobes and other types of bacteria carried out in this laboratory, the potentials registered by both platinum and gold-plated platinum electrodes have been recorded. The potential values with the two types of electrodes were usually found to run closely parallel courses (0.002 to 0.010 volts being the limits of variation commonly observed). The gold-plated electrodes were usually negative with respect to the platinum electrodes. The potentials at the two electrodes approached each other more closely as de-aeration (removal of atmospheric oxygen and metabolic gaseous elements) progressed.

For the potentiometric determinations, the circuit developed by DuBois[4] (1930) for use with the glass electrode was adopted. The calibrated potentiometer was the Leeds and Northrup

[4] We are indebted to Mr. DuBois, not only for very valuable advice and assistance in the construction and use of the potentiometer, but also for instruction in the preparation of the glass electrode.

Hydrogen-ion type. The galvanometer was a Leeds and Northrup cabinet type, with galvanometer (1000 ohm resistence), reflector and scale complete in one unit. It was sufficiently sensitive to permit readings to within less than 1 mv. The 3.5 N calomel half-cell, as recommended by Hewitt (1933), was used as the working standard. It was occasionally checked against 0.1 N, 3.5 N, and saturated calomel half-cells. The potentiometric apparatus proved to be very satisfactory and reliable, not only in the determination of oxidation-reduction potentials, but also in the accurate observation of the H-ion concentration with the glass electrode.

The following is a brief summary of the procedure followed in setting up an experiment. The bridges, with the tips drawn to capillaries, were filled and the tips sealed. Following the cleaning of the electrodes, the electrodes, bridges, gas connections, etc., were fitted into the vessel stoppers and the assembled stoppers and parts sterilized in the autoclave, with the platinum electrodes immersed in distilled water. The culture medium was placed in the electrode vessels, which were then cotton-stoppered and autoclaved. 75 cc. of medium were filled into each vessel. (The composition of the medium is given on graphs used to illustrate the results of the various experiments.) After sterilization, the medium in the vessels was cooled rapidly and inoculated. The vessels were assembled by transferring the stoppers bearing the electrodes, bridges, etc., to the culture vessels, with aseptic precautions. The stoppers were carefully sealed into place with gold size, and the vessels set up in the incubator. Gas connections were made with each vessel, the electrical connections were completed, and controlled de-aeration was begun immediately. The frequency with which readings were recorded depended somewhat upon the rapidity of potential change anticipated. Efforts were made to follow all significant changes. Occasionally, however, such changes escaped notice, because, as Hewitt also pointed out, even a scientific investigator requires sleep.

EXPERIMENTAL

The following isolated experiments are selected to illustrate most clearly the aspects of bacterial oxidation-reduction potentials which form the subject of this report.

Experiment 23

The most apparent observation made in this experiment, one which is readily reproducible, is that *Clostridium tetani* appears to produce and maintain a more intense final reduction potential than does *Clostridium botulinum*, and this in spite of the fact that cultures of *C. botulinum* grow more profusely and exhibit considerably more reducing capacity[5] than do cultures of *C. tetani.*

The conditions under which Experiment 23 was carried out are indicated in graph 1. Four cultures of a single strain of each of the two species, *C. botulinum* and *C. tetani*, were studied under very carefully controlled conditions with regard to the gaseous environment to which the cultures were exposed. The inoculum used in each case was 0.5 cc. of supernatant fluid from 48-hour egg-meat cultures.

The results are shown in graph 1. The potential differences which were established between the cultures of the two species are quite distinct. The experiment was not terminated after 4 days, as the graph indicates. Two cultures of each species were kept under strict anaerobic conditions until the end of the seventh day. At that time the potentials were still at the same levels, Eh −0.30 and −0.35 v. for *C. botulinum* and *C. tetani*, respectively. This difference in potential between these species was observed in 5 other similar experiments, one of which, Experiment 26, is described in detail here. Altogether, eight strains of *C. botulinum* and five of *C. tetani* were studied.

Experiment 26

This was planned primarily to corroborate, if possible, previous observations which indicated that the two species, *C. botulinum* and *C. tetani*, possess the property of establishing reduction potentials (under practically identical cultural conditions) which are significantly different; in other words, that the two species establish stable potentials which have differential value. Every effort was made to control experimental conditions, especially with respect to (1) supplying anaerobic conditions of a high degree

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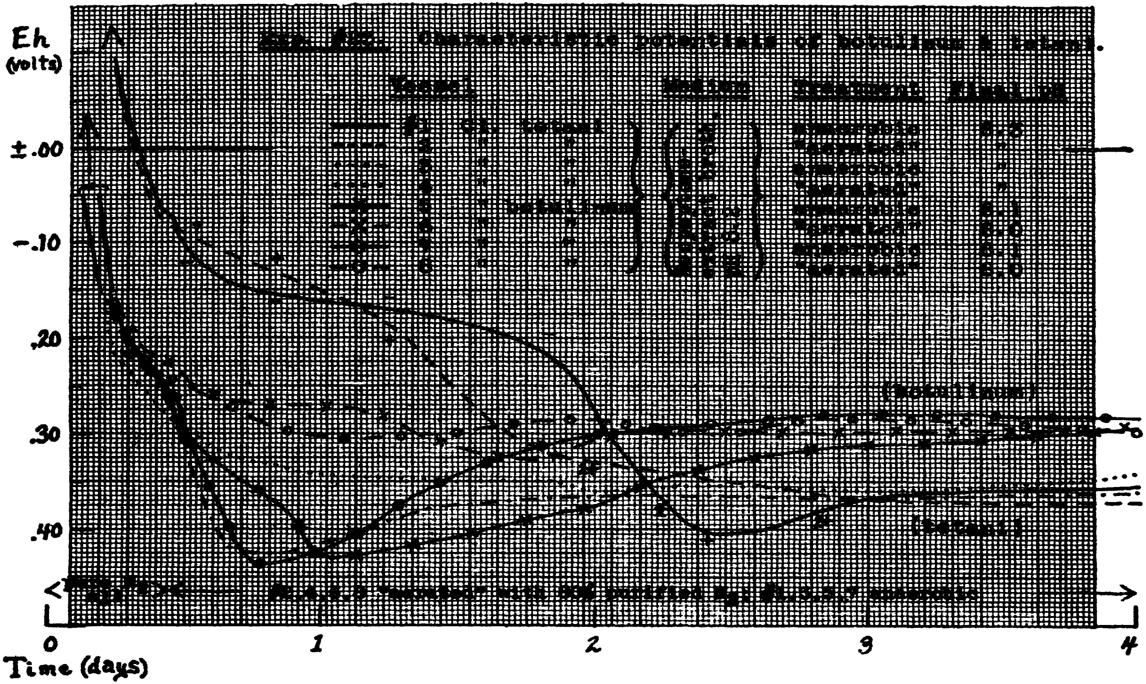
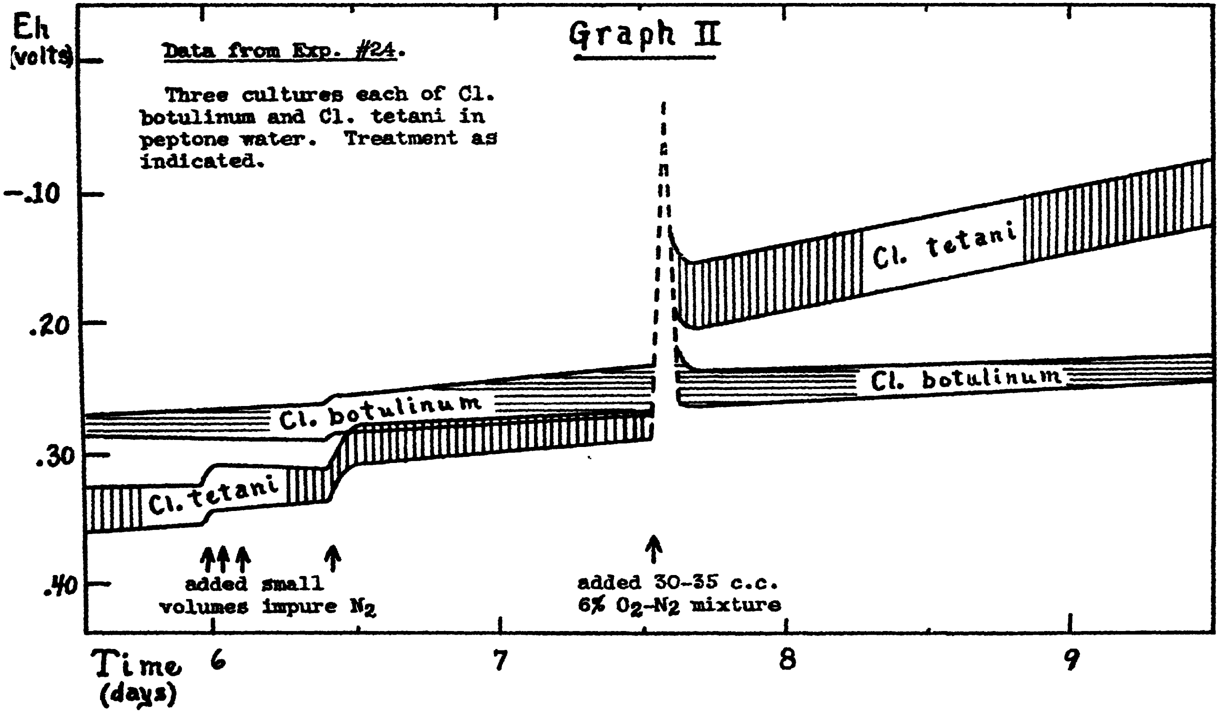
[5] When "aerated" with impure nitrogen, or richer oxygen-nitrogen mixtures, cultures of *C. botulinum* exhibited much more stability of potential than did cultures of *C. tetani*. This is illustrated in graph 2.



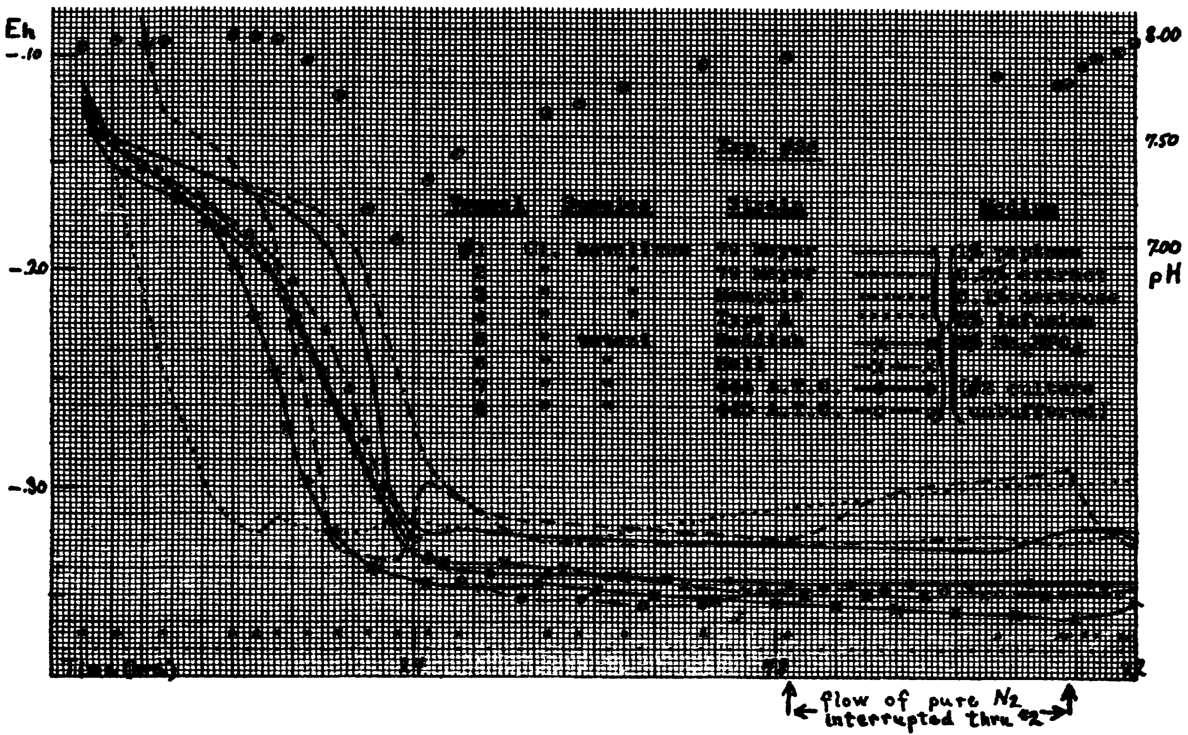
GRAPH 1

Note: + and − signs along curves 1 and 2 indicate development of turbidity due to bacterial growth in these two cultures. Linde tank nitrogen was used to deaerate the sealed culture vessels. All vessels were thoroughly flushed for 10 hours with nitrogen from which the oxygen impurity was removed (at least very substantially reduced) by passage over red hot reduced copper. The passage of gas was maintained through cultures #2, 4, 6 and 8. The gas used for this purpose was a mixture of 80 per cent purified nitrogen and 20 per cent nitrogen by-passed directly from the tank without removal of the oxygen impurity. It should be noted that the four cultures through which the passage of gas was continued reached stable potentials earlier than the other four cultures, probably as the result of this treatment. The trace of oxygen present in the nitrogen which was not purified had no obvious effect on the intensity of the potentials of these cultures, as compared with the four cultures which were not thus "aerated."



GRAPH 2

Illustration of relative reducing capacities of cultures of the two species (see Discussion)



GRAPH 3

Note: As indicated in the legend and in the description of Experiment #26, all cultures were buffered except #2 which is represented by the broken line. Progressive changes in H-ion concentration in culture #2 are recorded as circled dots at top of graph (see pH ordinates, upper right corner). Potential changes in cultures #1 and 2, which are identical except for the absence of phosphate buffer in #2, should be compared to observe the stabilizing effect of the buffer. Periods at which potential determinations were made are indicated by the crosses at the bottom of the graph.

throughout the experiment, (2) elimination of "hydrogen electrode effect" by continual passage of liberal amounts of purified nitrogen through the vessels, and (3) control of hydrogen-ion concentration by means of buffers. The essential data pertaining to the conditions under which the study was carried out are shown on graph 3.

The potential characteristic of the two species were reached early and remained stable until the experiment was terminated. The culture of *C. botulinum* in vessel #2 *was not buffered.* It was included in order to determine the relationship between O/R potentials and the pH changes recorded by a glass electrode immersed in the culture. This problem is to be made the subject of a subsequent report. However, the effect of the accumulation (theoretical) of metabolic carbon dioxide upon the Eh and pH of culture #2, which occurred when de-aeration was interrupted, and the effect of the subsequent removal of the carbon dioxide, are of interest.

The possibility that differences in pH may be wholly responsible for the final potential levels must be taken into account. The following figures are pertinent. Minor differences in reaction *did* develop in the cultures of the two species. The three buffered *C. botulinum* cultures had final reactions of pH 7.73, 7.73, and 7.67. The four cultures of *C. tetani*, similarly buffered, ranged from 7.82 to 7.83. However, to explain the difference in potential between the two species on the grounds of this difference in hydrogen-ion concentration would make it necessary that the ratio Eh:pH be of the order of about 0.28:1.00. This "slope" is considerably greater than what may be expected (see Clark and Cohen, 1923, 1, and Knight, 1930, 1).

DISCUSSION

The mechanisms which account for the potential changes in bacterial cultures are still imperfectly understood, largely, perhaps, because of our limited knowledge regarding the chemical constituents of the media used in bacteriology. In a sterile medium "in equilibrium" with its environment, the chemical mosaic would be reasonably well fixed. On the other hand, this mosaic would be kaleidoscopic during active bacterial growth. This simile may actually apply, since Cannan, Cohen and Clark (1926) conceive of the system present in culture media as consisting of a large electromotively inactive reserve, of which a limited part is progressively rendered electromotively active, by bacterial catalysis, in sufficient quantity to supply the energy and the cell-building requirements of the growing culture. This theory seems to be in accord with the observations recorded in the literature on bacterial O/R potentials. Since different bacteria are known to differ markedly in the intensity and extent to which they reduce various substrates, one is led to inquire into the possible existence of such differences between closely related bacterial species, and into the mechanisms of regulation and limitation of bacterial reduction.

In a few instances potential differences between widely different types of bacteria have been definitely correlated with distinct differences in metabolic activity. It is, therefore, conceivable that closely related species may possess individual potential characteristics. The observations reported here support such an assumption.

Preliminary studies by us with a number of other *Clostridium* species indicate definitely that *C. botulinum* and *C. tetani* are not unique in the possession of distinct and individual reducing properties.

Furthermore, evidence has been obtained by us (subsequent publication) of the occurrence of distinctly different potentials between species or types of (1) lactobacilli (Gillespie and Rettger, 1936, abstr.), (2) non-spore-forming obligate anaerobes of the *Fusobacterium*[6] genus, and (3) members of the genus *Aerobacillus* (Gillespie and Porter, 1937, abstr.). The work of Sagen, Riker and Baldwin (1934) and Burrows and Jordan (1935 and 1936) may also be cited as further evidence.

This manner of reasoning may be extended also to include strains within a given species, since strains have been known to

[6] Experiments with fusobacteria were carried out in coöperation with Dr. Earle H. Spaulding.

possess individual traits. However, we may be safe in assuming that strain differences rarely, if ever, result in the development of final potential differences of any great magnitude. Furthermore, traits characteristic of individual strains are often considered to be of a variable nature.

We can only attempt to explain the discrepancy between the results obtained by us with the spore-forming anaerobic group, and those reported by Plotz and Geloso (1930), on the basis that the experimental conditions used in the two laboratories were different. Plotz and Geloso held their cultures *in vacuo.* One result of their method may have been effective elimination of gaseous metabolites. Although the conditions under which our studies were made would also tend to eliminate gases resulting from metabolism, the possibility remains that such products may have influenced the results. Considerable evidence has accumulated that certain of the gases liberated during bacterial activity do, through one mechanism or another, affect the potentials of metallic electrodes employed to record O/R conditions (Lepper and Martin, 1930 and 1931, Boyd and Reed, 1931, and others).

It is interesting to contrast the effects of capacity and intensity, as featured in the reductions which occur in cultures of *C. tetani* and *C. botulinum* (see graph 2); the effects are diametrically opposed. *C. tetani* cultures exhibit higher reduction intensity and much lower capacity than do *C. botulinum* cultures. Thus, the possibility of attributing the potential difference which separates these two cultures to the effect of capacity of the medium is eliminated.

Various attempts have been made to relate the extremely negative potentials which are temporarily exhibited in cultures of certain bacteria, notably those which form hydrogen, to the influence of this gas upon the electrode. Cannan, Cohen and Clark (1926) offer the logical suggestion that one would expect hydrogen to be liberated only at potentials of hydrogen overvoltage. Lepper and Martin (1930 and 1931) offer a theory to explain the phenomenon of hydrogen overvoltage, namely, that supersaturation of the substrate with hydrogen may occur because of the conditions under which the gas is liberated.

We have found that effects which may be attributed to metabolic hydrogen can be eliminated by continual passage of purified nitrogen through the culture. In this way the extreme negative potential "peak," of short duration, which occurs in cultures not subjected to continual de-aeration may be eliminated; the potentials become stabilized at an early stage of development and remain at a constant level.

It would hardly be safe to assume that any single phase of physiological activity can be completely responsible for all peculiarities of potential which may be observed during the most active period of growth of bacterial cultures. In fact, the evidence points to the contrary. For instance, the most pronounced and rapid changes in hydrogen-ion concentration usually occur at this stage of development of the culture. Certain potential fluctuations can be attributed to the influence of such pH changes (Gillespie and Rettger, 1936, abstr.). Moreover, it is in this period that chemical conditions are in a most unstable state. The nature of the metabolites, the interrelationship between metabolic reactions, the speed with which they progress, and the rapidity with which the electrodes reach stability must all receive consideration.

It becomes apparent that any attempt to analyze the picture presented by the potential-time history of bacterial cultures must be undertaken with painstaking consideration of the many variables which may affect results. Certain of these variables, such as control of H-ion concentration and the elimination of the influence of atmospheric oxygen, can be effectively regulated. Others, still more intimately connected with the manner in which an organism reacts to its environment, have yet to be determined. One element of confusion in such studies is associated with the fact that the various physiological and chemical changes which occur as the result of bacterial activity reach equilibrium only after an ill-defined period, and that protoplasmic adaptability allows for considerable flexibility in the interrelationship between various physiological processes.

CONCLUSIONS

Evidence has been presented that *Clostridium botulinum* and *Clostridium tetani* establish final potentials (in about 2 or 3 days at 37°C.) at significantly different levels of reducing intensity, levels which may be considered as characteristic of the species.

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BACTERIAL OXIDATION-REDUCTION STUDIES

II. Differentiation of Lactobacilli of Diverse Origin[1]

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Received for publication May 31, 1938

INTRODUCTION

Few attempts have been made to compare O/R potentials exhibited by closely related bacterial species. The observations of Sagen, Riker, and Baldwin (1934) and Burrows and Jordan (1935 and 1936) are cited in the first paper in this series. We were successful (Gillespie and Rettger, 1938) in distinguishing some of the well-known spore-forming anaerobes from each other on the basis of stable reduction potentials of different intensity. The earlier results of our studies with respect to definite O/R potentials characteristic of species within the *Clostridium* and *Lactobacillus* genera were reported in the doctorate dissertation (1935) of the senior author. Evidence of the ability of species within other genera, namely *Aerobacter* and *Fusobacterium*, to establish potentials of decidedly different intensity has also been obtained in this laboratory (Gillespie and Porter, 1937, abstr. and Gillespie and Spaulding, unpublished data).

It is the purpose of this paper to present data which strongly indicate that certain lactobacilli exhibit stable reduction potentials which differ strikingly in degree of intensity. In our estimation, these potential differences may have species differentiation significance.

APPARATUS AND METHODS

Apparatus and technique were devised which enabled us to study O/R potentials and related phenomena in bacterial cultures

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[1] The cost of the original equipment used in this work was met by a grant from the Fluid Research Fund of the Yale University School of Medicine.

under conditions which can be strictly controlled.[2] Potentials were determined by means of the circuit devised by DuBois (1930). The electrodes were of coiled platinum wire.[3] The cultures were grown in electrode vessels within which strict anaerobic conditions were established and maintained by continuous flow of purified nitrogen. Small volumes (0.5 to 1.0 per cent) of carbon dioxide were mixed with the nitrogen. Changes in potential which occurred in the deaerated cultures were followed by readings made at frequent intervals. Changes in H-ion concentration were either sufficiently suppressed, for all practical purposes, by means of buffers, or closely and accurately followed by means of glass electrodes. Sterile medium controls were included in the experiments, in order to determine the characteristic potentials of the various media employed.

Two types of media were used in these experiments. The first, a broth containing tomato juice (Rettger, Levy, Weinstein and Weiss, 1935) is particularly well adapted for culturing *Lactobacillus acidophilus* and related species. This medium could not, however, be effectively buffered when used for this purpose. Therefore, a second, highly buffered, medium was developed for use in determination of the characteristic potentials of the lactobacilli at relatively constant pH. The effect of changes in H-ion concentration upon the reduction potentials of these highly fermentative organisms and other types of bacteria is the subject of another report (Gillespie, unpublished data).

EXPERIMENTAL

The experiments presented here are representative of many in which practically identical results were obtained.

Experiment 30

The composition of the medium used for culturing lactobacilli in this experiment was as follows: (*See next page*)

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[2] The apparatus and methods are described in full in the senior author's Ph.D. dissertation deposited in the Sterling Library of Yale University.

[3] Comparison was made later between platinum and gold-plated platinum electrodes. The differences in potential recorded with the two types of electrode were not of such a magnitude as to affect appreciably the results reported here.

Tomato juice	20 per cent
Peptonized milk	1 per cent
Yeast extract	0.5 per cent
Peptone	0.5 per cent
Initial pH (after sterilization)	7.1–

The following organisms were employed: Strains *L* and *R*—typical oral lactobacilli; *B-16*—*Lactobacillus bulgaricus*; *M-3*—isolated from the human vagina and classed as *Lactobacillus doederlein*; *MI*—a strain of unknown origin, but from all indications not an oral type.

Practically all of the strains used in this work had been subjected to exhaustive taxonomic study in this laboratory.

The culture vessels, each containing 75 cc. of medium,[4] were inoculated from three-day-old tomato broth cultures: 1 cc. was used as inoculum for the buccal strains, and 5 cc. for the others.

The electrode vessels were assembled, sealed with gold size and placed in an incubator at 37°C., where gas and electrical connections were completed. Sterile glass electrodes were introduced, with aseptic precautions, into electrode-culture vessels #1 and #2. The cultures were rapidly and thoroughly deaerated with purified nitrogen. Oxidation-reduction potentials (and glass electrode potentials in cultures #1 and #2) were determined with sufficient frequency to detect significant changes.

The results of Experiment 30 are illustrated on graph 1. In spite of the very obvious influence of changes in H-ion concentration (increase in H-ion concentration causes the potentials to shift to levels of lower apparent intensity), maximum reduction potentials were established which distinguish the two strains of oral origin from the other three strains by a wide margin.

H-ion concentration changes in the two cultures of oral lactobacilli were followed at frequent intervals by means of the glass electrodes inserted and sealed into the vessels. In table 1 are listed the progressive changes in Eh and pH which were recorded in these two cultures (#1 and #2). The correlation between

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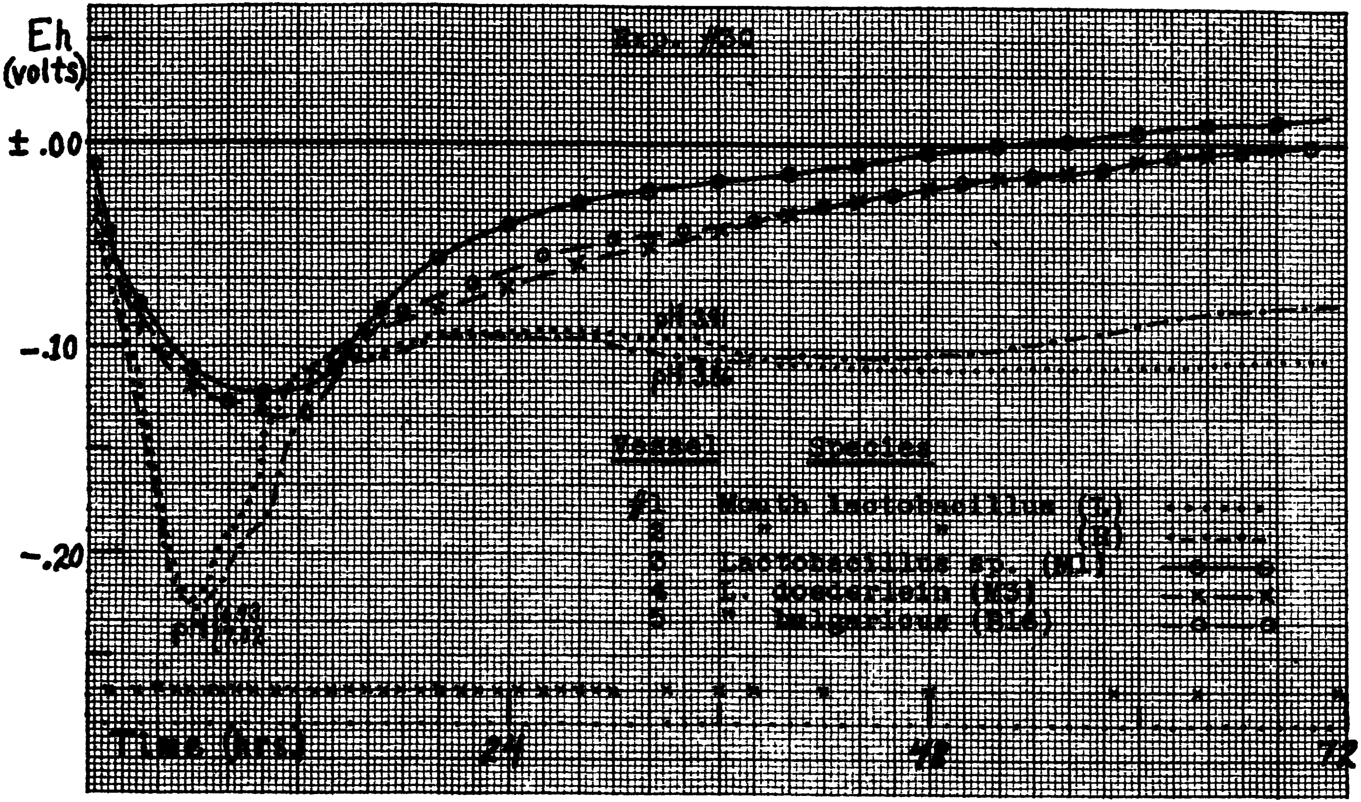
[4] Media used in all O/R studies were prepared and autoclaved immediately before the experiments were set up. This practice was followed in order that the medium might be exposed to the oxidizing influence of air for as short a period as possible.



GRAPH 1. EXPERIMENT 30

Note: The cross marks at the bottom of the graph indicate periods at which Eh and pH readings were made the changes in pH and the simultaneous O/R potential trends is obvious.

No pH determinations were made on the cultures of the *MI*, *M3* and *B-16* strains before the end of the experiments. We feel certain, however, that the gradual and continual potential trend in these cultures toward an apparently more highly oxidized state can be definitely attributed (as was previously determined) to continued fall in pH. In other words, our experience indicates very definitely that the corresponding Eh and pH changes in cultures of *L. acidophilus*, *L. doederlein* and *L. bulgaricus* (cultures #3, 4 and 5) would be of the same order. The reactions of the cultures when the experiment was terminated (at 72 hours) were as follows: pH 3.94 and 3.90 for the two oral strains, and pH 4.37,

TABLE 1

Experiment 30. Eh and pH changes recorded in cultures #1 and #2 (oral lactobacilli)

HOURS	CULTURE #1		CULTURE #2	
	Eh	pH	Eh	pH
0	−.035	7.03	−.026	7.06
1	.068	7.05	.063	7.07
2.5	.113	7.07	.111	7.10
3.75	.180	7.04	.176	7.09
4.75	.213	7.00	.216	7.07
5.5	.221	6.93	.225	7.02
6.25		6.73		6.81
6.5		6.49		6.62
7.25	.192	6.28	.167	6.45
8.25	.182	5.97	.191	6.27
10.5	.128	4.87	.160	5.34
12.5	.106	4.44	.118	4.55
14.5	.103	4.26	.106	4.22
17.5	.096	4.13	.099	4.06
21	.091	4.04	.092	3.94
26.5	.089	3.93	.094	3.89
31.5	.093	3.91	.101	3.86
46.5	.108	3.94	.102	3.89
71	.096	3.94	.075	3.87

For the sake of brevity not all readings are listed. Sufficient data are tabulated to demonstrate the marked changes and the definite relationship.

4.54 and 4.39 for strains *MI*, *M3* and *B-16*, respectively. Had the H-ion concentration been the same in all five cultures during the experiment the differences in potential between the two types would have been still greater. This statement is supported by the results of Experiment #29 (below) and other experiments, not reported here. A more comprehensive discussion of the Eh:pH relationship in bacterial cultures will be presented in a future publication.

Experiment 29

In order to demonstrate the characteristic potentials of the two types of lactobacilli still more clearly, potentiometric studies were made, using a highly buffered culture medium. Under these conditions the influence of pH change was largely eliminated, and stable reduction potentials at two widely separated levels were established. The composition of the medium is given on graph 2. The initial reaction was pH 7.1.

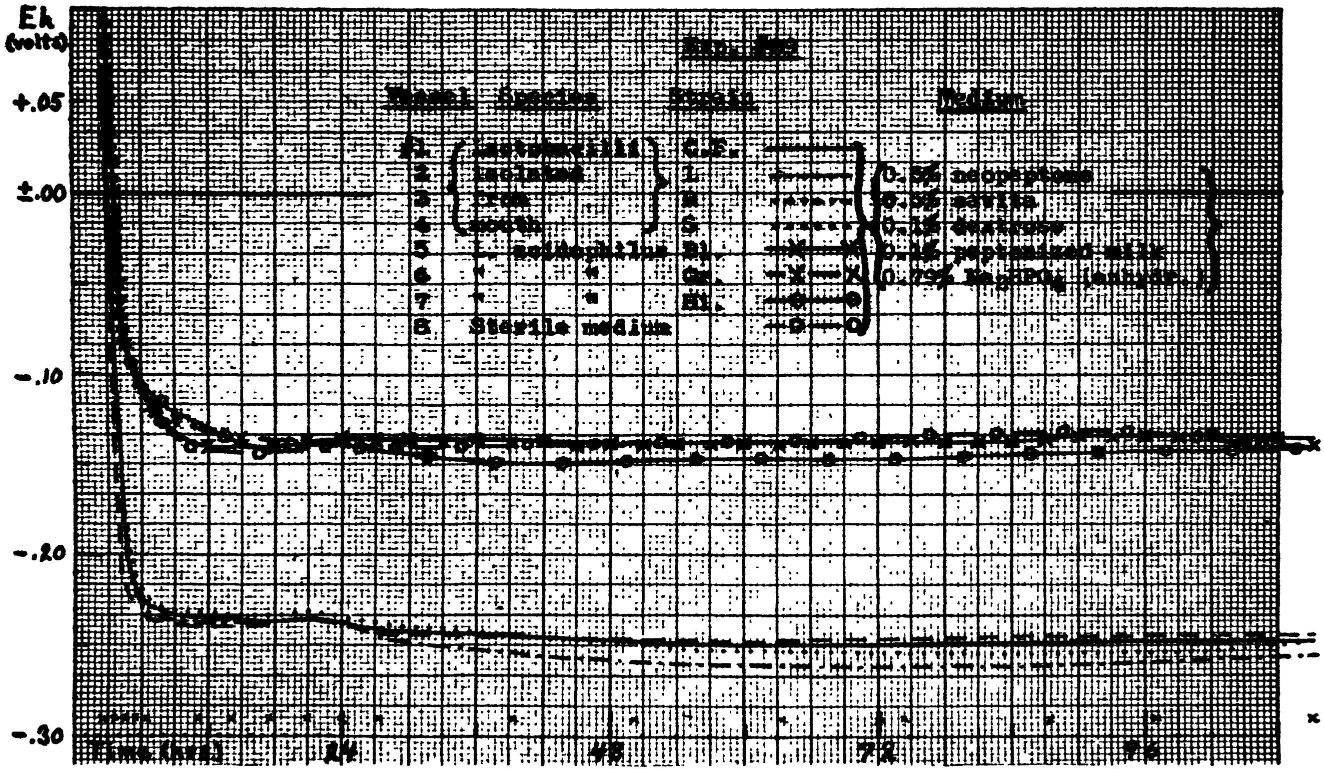
The seven strains studied include four of oral origin (typical mouth strains), *CF*, *L*, *R*, and *S*, and three typical *L. acidophilus* (intestinal) strains, *Bl*, *Gr*, and *Hi*. The potential changes which occurred in the sterile medium control were also determined.

The electrode-culture vessels were inoculated from 1-day-old tomato broth cultures: 1.5 cc. of inoculum was used for the oral strains, and 7.0 cc. for the strains of *L. acidophilus*.

The vessels were set up, sealed and placed in the incubator, as described in Experiment 30 (above). The results are shown on graph 2.

The four *Lactobacillus* strains of oral origin established maximum reduction potentials of decidedly greater intensity than did the three *Lactobacillus acidophilus* strains. The difference in potential levels exhibited by these two types was 0.10 and 0.11 v. or more. This difference became apparent early in the experiment and was maintained until its termination. Only minor differences in potential developed between the strains within each group. These minor degrees of reducing intensity may be indicative of strain characteristics.

The fall in pH which occurred in the seven cultures in the



GRAPH 2. EXPERIMENT 29

Note: The cross marks at the bottom of the graph indicate periods at which Eh and pH readings were made buffered medium, during the 4½-day period covered by the experiment, ranged from 0.4 to 0.65 unit. Although the changes in reaction were sufficient to have significant influence upon the O/R potentials, it appears from a comparison of graphs 1 and 2 that the effect was of decidedly reduced magnitude in the latter instance.

Results similar to those just described have been obtained repeatedly. Lactobacilli of known oral origin, or exhibiting the characteristics of the oral type, were found invariably to possess highly active reducing tendencies, as compared to strains of intestinal origin and similar types, including *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus bifidus*, and Doederlein's bacillus.

The potentiometric experiments were supplemented by studies in which a colorimetric technique was employed for the determination of reduction intensity. When the colorimetric method was used the lactobacilli of the intestinal type sometimes showed a mild tendency to produce a reduced state of intensity greater than that acquired by the anaerobic sterile medium. With respect to the degree of *rapidity* and *intensity* of reducing activity, however, the intestinal type lactobacilli never approached the oral type. A total of 74 *Lactobacillus* strains was studied.

DISCUSSION

The theory that bacterial O/R potentials are influenced, if not determined, by changes in the chemical complexity of the culture medium which are brought about through bacterial metabolism is not generally accepted. It seems that this theory should, in the case of the lactobacilli, account for the striking differences in reduction intensity which, according to our observations, are characteristic of types or species. Consideration of the known facts regarding the purely *qualitative* aspects of the metabolism of the oral and intestinal types of lactobacilli leads one to conclude that there is no correlation between characteristic potentials and metabolism.

Strains of the oral type appear to be much more active than those of the intestinal type, with respect to both rapidity and intensity of growth. The difference is indicated by earlier and more marked development of turbidity, greater rapidity in progress of fermentation and earlier development of highly reduced conditions in cultures of the oral type. It appears, therefore, that there may be a correlation between the intensity of bacterial activity (that is, the *quantitative* aspect of metabolism) and the O/R potentials in the case of the lactobacilli. Certain of our potential studies tend to support this theory; on the other hand, other evidence which has accumulated seems to be contradictory.

A limited amount of work was done in attempting to answer this question. Among our cultures were two strains which approached the oral type in rapidity and intensity of growth, fermentative activity, etc.; yet their potentials were of the same magnitude as those established by typical *L. acidophilus*. Assuming that the media used for culturing these organisms may have sufficient capacity to limit the bacterial reducing activity, particularly when the bacterial activity is relatively mild, as is the case with the intestinal type, we have attempted to reduce the capacity factor by reducing the concentration of the various ingredients of a favorable medium. The results were equivocal. Attempts to bring the hypothetical poising system or systems to a delicate balance by titration with reductants likewise failed, partly due to technical difficulties. Determination of pH changes and rough estimation of changes in turbidity were the only measurements of metabolism which were applied in these studies. A more thorough study of physical and chemical changes in the cultures, in conjunction with potential readings, might indicate what phase of metabolism determines the potential difference which we have observed between lactobacillus types.

Ordinarily (except as mentioned above) the intestinal lactobacilli showed no tendency to carry the potential below the level that characterizes the sterile medium. In such instances, effects due to bacterial oxido-reductive activity and those due to autoxido-reductive effects inherent in the medium cannot be distinguished. Certain observations made in connection with this phenomenon suggest the existence of a certain amount of poising capacity in the medium (Gillespie, 1936, abstr.). The theoretical, or perhaps more exactly, hypothetical, effect of capacity appears to be most active at the potential level characteristic of the sterile medium. In fact, if such an influence actually exists, it perhaps determines the potential of the sterile medium under anaerobic conditions. This aspect of the work constitutes a problem in itself, and is by no means definitely settled.

With regard to the intensity of the reducing activity of *L. acidophilus*, our results differ from those reported by Longsworth and MacInnes (1936). Two possible explanations may be offered for the lack of agreement. First, no generally accepted methods have, at least until quite recently, been forth-coming for classification of the lactobacilli, in-so-far as differentiation of oral, intestinal, and other closely related types and species is concerned. Second, the conditions under which the experiments were carried out in the two laboratories were very different, particularly with regard to composition of media and cultural conditions. The medium used by Longsworth and MacInnes would, we believe, support a much more luxuriant growth of *L. acidophilus* than our own, the sugar content of which was intentionally reduced to a minumum in order to avoid increase in H-ion concentration of any great magnitude. The discrepancy in final reduction potential is, therefore, conceivably associated with failure of *L. acidophilus* cultures in the experiments described above to overcome the poising capacity of the medium. However, we consider the problem unsettled.

CONCLUSIONS

Experimental evidence presented in this paper indicates that lactobacilli of oral origin can be differentiated sharply from *Lactobacillus acidophilus* and closely allied species upon the basis of intensity of reducing activity. Oral lactobacilli were found to establish much more intense reduction potentials than *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus bifidus* and Doederlein's bacillus. The characteristic potentials of the two types differed by as much as from 0.10 to 0.11 volts. It appears that a correlation may exist between the O/R potential of these two groups and the degree of intensity of their metabolic activity.

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BACTERIAL OXIDATION-REDUCTION STUDIES

III. CHARACTERISTIC POTENTIALS OF CULTURES OF AEROBACILLUS[1] SPECIES[2]

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Received for publication May 31, 1938

INTRODUCTION

In other papers from this laboratory three instances are reported of marked differences observed in the oxidation-reduction potentials of closely related species or types of bacteria in widely different genera. Differential levels were determined for *Clostridium botulinum* and *Clostridium tetani* (Gillespie and Rettger, 1938 (1)), for lactobacilli of oral and intestinal origin (Gillespie and Rettger, 1938 (2)), and for different types of fusobacteria (Gillespie and Spaulding, unpublished). The present paper marks the fourth successful attempt in the laboratory to demonstrate final reduction potentials of widely different magnitude of intensity in closely allied bacterial species. It deals with the O/R potentials of two species of the *Aerobacillus* genus, namely *Aerobacillus polymyxa* and *Aerobacillus macerans*.

The results of these observations add further evidence that differences of potential between closely related types and species may be regarded as significant, from the standpoint of bacterial classification.

A review of the literature appears in the first publication of this series. It reveals very few comparative oxidation-reduction studies on closely related species within given genera.

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[1] The genus *Aerobacillus* was created in 1926 by Donker, to include all sporulating, facultative anaerobic bacteria which produce catalase and are able to produce acid and gas from various carbohydrates. Five species were recognized by Donker.

[2] The cost of the original equipment used in this work was met by a grant from the Fluid Research Fund of the Yale University School of Medicine.

METHODS

The apparatus and methods used to carry out these studies are described elsewhere in some detail.[3] Platinum and gold-plated platinum electrodes, in conjunction with a vacuum tube potentiometer circuit, were used for the determination of potentials. The cultures were grown in electrode vessels, within which strict anaerobic conditions were established and maintained by continuous flow of purified nitrogen.

The composition of the medium is given on the graph. It was such as to support rapid and prolific growth of the aerobacilli. It was effectively buffered. Changes in reaction in cultures over 4 to 5-day periods did not exceed 0.1 unit pH. The potential drift which occurred in the de-aerated cultures was followed until relatively stable levels were reached, and thereafter for several days. These stable levels of potential should, we believe, have differential significance.

EXPERIMENTAL

The genus *Aerobacillus* has been reported (Porter, McCleskey and Levine, 1935, 1937) to be rather sharply divided into two definite species, *Aerobacillus polymyxa* and *Aerobacillus macerans*, on the basis of certain cultural, biochemical and serological characteristics. The species are defined chiefly, first, on the basis of growth temperature limits; second, on ability to ferment rhamnose and sorbitol, with acid and gas production; third, on the production of acetyl-methyl-carbinol; and fourth, on the basis of agglutinative antigen components. The striking physiological differences suggested a study of oxidation-reduction potentials of representative strains of the two species.

Graph 1 illustrates the very close correlation which was found to exist between the groups as established by Porter, McClesky and Levine, and the one arrived at in the potentiometric studies.

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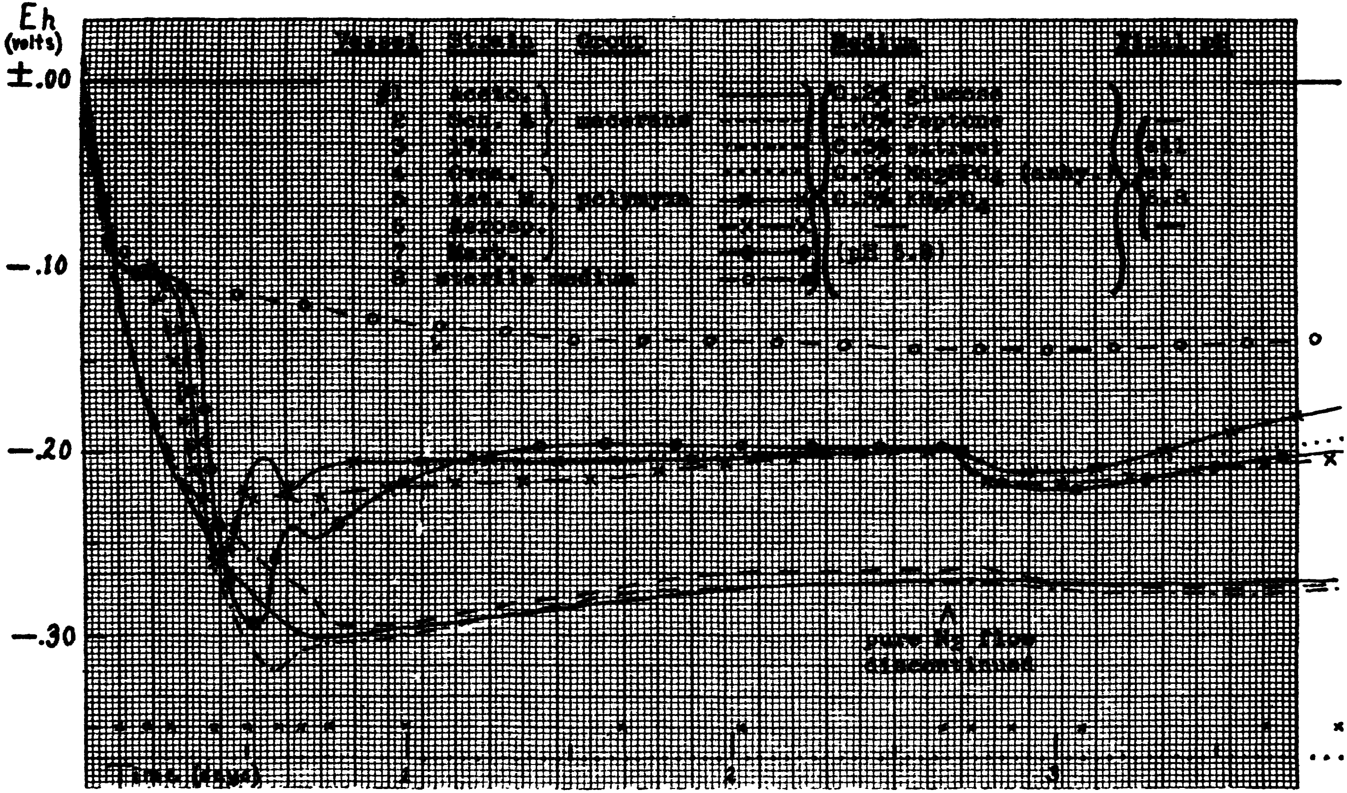
[3] Senior author's Ph.D. dissertation on file in the Sterling Library, Yale University. For brief description see first paper in this series, this Journal, 1938.



GRAPH 1. EXPERIMENT 46

Note: The crosses at the bottom of the graph indicate periods at which potential readings were made

Potential-time curves of anaerobic cultures of seven strains of aerobacilli are shown. The experiment was repeated under practically identical conditions, with the same results. In all, six strains of "*macerans*" and eight of "*polymyxa*" were studied. All of the six strains of the "*macerans*" species employed showed a marked difference in final reduction potential from the eight "*polymyxa*" strains—a difference of at least 60 to 70 millivolts. Here again, strains within a given species or group were found to show only slight variations, as compared with the strains in the two different species or groups.

DISCUSSION

There is now a general tendency to accept the theory that the potentials which develop in bacterial cultures are influenced, not to say established, by the definite type of metabolic activity of the bacteria concerned. While this theory seems to be reasonably well founded, attempts to correlate potentials with the existence of specific reversible O/R systems in the substrate have been, as a rule, not more than suggestive, at best. The work reported here points to interesting possibilities. The probability of a direct correlation between the growth temperature limits of these two groups, or between their ability to ferment rhamnose and sorbitol, on the one hand, and the characteristic potentials which they exhibit on the other, appears to be definitely excluded. The potential studies were carried out at 37°C., with neither rhamnose nor sorbitol present in the culture medium.

Burrows and Jordan (1936) offered the hypothesis that a correlation may exist between reduction potentials and antigenic structure of organisms. This, of course, necessitates consideration of antigen synthesis through metabolism. The potentials of several strains of the serologically heterogeneous polymyxa group (eight strains were studied) do not support this hypothesis. It would be surprising if such an hypothesis were universally applicable, considering the probable complexity of the conversion, extracellular and intracellular, of nutrient constituents of a medium into bacterial antigens. The fourth possibility indicated by the known facts in the case of the aerobacilli is that carbohydrate metabolism may be involved. That is, the two species of *Aerobacillus* appear to attack glucose differently, as is indicated by the Voges-Proskauer reaction. We hope to seek further for an explanation of the difference in reduction potential which these two groups display.

CONCLUSION

According to the observations reported here, the two known species of the *Aerobacillus* genus exhibit stable potentials at two widely different levels of reducing intensity. The grouping established potentiometrically coincides exactly with that previously established on cultural, fermentative and serological grounds. The experimental evidence suggests the possibility that differences in carbohydrate metabolism may be responsible for the difference in reduction potentials.

This is the fourth bacterial genus with which similar correlations have been established in our laboratory. The results of these experiments are such as to direct the course of further investigation into the significance of bacterial oxidation-reduction potentials.

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STUDIES ON STEAM STERILIZATION AND THE EFFECTS OF AIR IN THE AUTOCLAVE

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Received for publication August 1, 1938

Underwood (1937) has recently emphasized a number of the limitations of autoclave sterilization in a series of very practical articles. The work herewith reported involves experiments upon the detrimental effects on efficient steam sterilization of air in the autoclave.

An autoclave of special design was employed. This apparatus consisted of a small double-jacket sterilizer with separate boiler; the inlet and outlet valves were large so that pressure could be built up or released within 10 seconds. A total time of 30 seconds sufficed for the evacuation of over 99 per cent of the air and the attainment of the desired pressure of saturated steam,—a fact that was verified by numerous experiments. Figure 1 shows a diagram of the lay-out.

In order to run tests involving known mixtures of air and steam the discharge line of the autoclave was connected to a modified pneumatic trough and in this way the contents could be discharged through water, the steam condensed and the air measured by displacement. Since part of the air contents remained in the sterilizer even after it had reached atmospheric pressure following the first discharge of air and steam into the pneumatic trough, it was necessary to sweep out the remaining air by means of more steam before making the final measurement. This use of saturated steam to flush out residual air had the disadvantage of exerting an additive sterilizing effect which was quite marked at high pressures, under which conditions it often sufficed to sterilize spores which should otherwise have remained viable. In experiments at 20 pounds pressure with no air discharged from the autoclave it was found possible to dispense with this complicating factor and to measure the air only at the beginning and end of each series of tests. In other experiments at lower pressures, however, the air was evacuated and measured at the end of each run before the test swabs were removed from the autoclave.

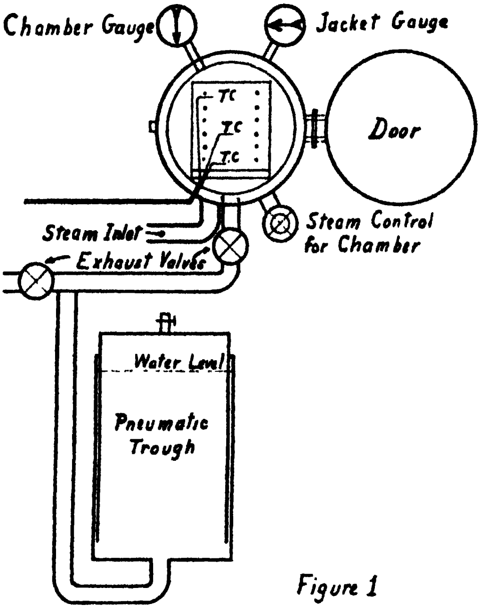

FIG. 1. DIAGRAMMATIC DRAWING OF AUTOCLAVE

The boiler is not shown. The *Steam Control for Chamber* represents a valve which passes steam from the jacket into the chamber. The jacket is always directly connected with the boiler, so that the *Jacket Gauge* represents boiler pressure. The boiler and jacket can carry a full head of steam with the chamber empty. The rectangle within the chamber represents a rack designed to carry two rows of test swabs placed horizontally at six different levels indicated by the two rows of dots. "*T. C.*" shows the approximate positions of the three thermocouple leads within the chamber. The left *Exhaust Valve* is shut and the right *Exhaust Valve* opened when one desires to empty the autoclave through the pneumatic trough. Both valves are opened to empty the autoclave past the pneumatic trough. The *Steam Inlet* opens into the middle of the back of the chamber. The exhaust valve opens from the bottom of the front of the chamber.

The percentage of air in the steam was calculated as follows: The air was actually measured at room temperature and pressure in the pneumatic trough; the volume it would occupy at the temperature and pressure existing in the autoclave was then calculated by means of the gas laws. The amount of air in the steam was then expressed as a percentage,—for instance, 25 per cent air means that at the temperature and pressure in the autoclave the steam and air are respectively 75 per cent and 25 per cent by volume.

The following is a sample calculation for air volume. The air collecting cylinder was of the same diameter as the inside of the autoclave and the autoclave chamber had a total length of 20 inches. Hence 5 inches of air in the cylinder would be $\frac{5}{20}$ of the total volume of the autoclave chamber. At 10 pounds gauge pressure and a temperature of 110°C. this $\frac{5}{20}$ of the chamber volume would be changed to

$$\frac{5}{20} \times \frac{14.7 \text{ lbs.}}{10 \text{ lbs.} + 14.7 \text{ lbs.}} \times \frac{273^\circ + 110^\circ}{273^\circ + 25^\circ} \times 100 \text{ per cent or } 19 \text{ per cent}$$

The temperatures within the autoclave were measured by means of three thermocouples which were customarily located at three different levels, one near the top, another one in the center and a third close to the bottom of the chamber. The thermocouples could be connected in turn by means of a multiple switch to a direct reading galvanometer. By maintaining the temperature of the constant junction at 100°C. in boiling water the temperature could be read quickly and accurately. When any considerable percentage of air was retained in the autoclave the lower thermocouple would always record a temperature of a few degrees below that registered by the upper two which were in close agreement. In this case the temperature for correcting the air volume reading was taken from the center thermocouple.

Clostridium oedematiens was employed as the main test organism. This bacterium is considered by a number of observers to be one of the most heat-resistant of any of the spore-forming organisms of surgical importance, an observation which was confirmed by one of us (Hoyt, 1934). Two strains were obtained from the American Type Culture Collection (*C. oedematiens*, No. 681 and *C. novyi*, No. 3539). Both liquified gelatin but failed to digest coagulated serum. Both fermented glucose, dextrin and maltose with the production of acid and gas and both failed to ferment mannitol, salicin, lactose and sucrose. *C. novyi* produced acid and gas in glycerol while *C. oedematiens* formed acid only. *C. oedematiens* proved more virulent for mice than did *C. novyi.* Their resistance to heat was, for practical purposes, identical when both organisms were employed in a number of the earlier experiments. In later work *C. oedematiens,* No. 681 was generally utilized but we have considered both organisms as different strains of *C. oedematiens* and they are designated as such in our experimental results.

A local strain of *Clostridium botulinum A* was also employed, mainly to check the experimental technic against a spore-former of extremely high heat resistance.[1] The resistance of *Clostridium welchii* and *Clostridium tetani* was also tested in a few instances.

All bacteria were grown for a minimum of 3 days in chopped-veal infusion broth and each batch of cultures was checked microscopically for spore formation before being utilized in an experiment. Sterile swabs were dipped into these tubes and then usually inserted into a rack which held 12 swabs, the rack was placed in the autoclave and the swabs were directly exposed either to saturated steam or to mixtures of steam and air. At the end of the period of sterilization, the autoclave was rapidly emptied of steam and each swab was removed and cultured in a tube of chopped-veal infusion broth which had recently been boiled and allowed to cool; the swabs were then discarded and the cultures were overlaid with sterile melted vaseline. Each culture was labeled with the date and the position of its swab in the autoclave. Control cultures of unheated organisms were made at the time of each experiment.

The cultures were incubated at 37°C. for a minimum of one month before being discarded. This was an arbitary time limit and we realize from the work of Dixon, Burke, Beck and Johnston (1925) and other reports that occasional spores may lie dormant for much longer intervals than this even when placed in a medium suitable for growth. In our results occasional tubes inoculated with *C. botulinum A* spores showed growth only after being incubated for periods ranging up to a month or more but the *C. oedematiens* cultures almost never became positive after an interval of more than 12 days.

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[1] We gratefully acknowledge the coöperation of Dr. Ivan C. Hall of the School of Medicine, University of Colorado in checking this culture for us.

Growth in the cultures was clearly evidenced by the production of gas which forced the vaseline away from the surface of the liquid. Smears were made from positive tubes chosen at random from time to time and stained by Gram's method to check the cultures for correct morphology. Very occasional cultures showed irregular evidences of growth, usually with a minimum of gas production; smears made from most of such tubes disclosed contaminants, usually staphylococci or diphtheroids. All tubes shown to contain contaminants were omitted from the tabulations of experimental results. Experiments were performed on 40 different days and involved a total of over 3,000 cultures exclusive of unheated controls.

EXPERIMENTAL RESULTS

Saturated steam served as the sterilizing agent in the earlier experiments and pressures of 6, 10, and 15 pounds corresponding to temperatures of 110, 115, and 121°C. were employed. These temperatures within various parts of the autoclave were checked by thermocouples during every test and variations from the values indicated were negligible. Tables 1, 2 and 3 summarize 24 experiments upon the sterilizing effects of saturated steam on spores of *Clostridium botulinum A* and *Clostridium oedematiens.*

Tables 1, 2 and 3 indicate that a direct contact of saturated steam with spores of *C. botulinum A* and *C. oedematiens* required the following minimum times to kill these organisms completely:

	C. BOTULINUM A	C. OEDEMATIENS
	minutes	*minutes*
6 pounds saturated steam, 110°C.	Over 30	10
10 pounds saturated steam, 115°C.	10	4
15 pounds saturated steam, 121°C.	4	1

TABLE 1

6 pounds saturated steam, 110° C.

C. BOTULINUM A			TIME OF EXPOSURE	C. OEDEMATIENS		
Total cultures	Cultures showing growth	Percentages of cultures showing growth		Total cultures	Cultures showing growth	Percentages of cultures showing growth
			minutes			
30	30	100	2½	24	24	100
			4– 5	45	38	84
36	36	100	6	41	36	88
			7½– 8	84	12	14
			10	69	0	0
126	102	75	11–15	36	0	0
108	67	62	16–20	12	0	0
18	3	17	21–25			
12	1	9	30			

TABLE 2

10 pounds saturated steam, 115°C.

C. BOTULINUM A			TIME OF EXPOSURE	C. OEDEMATIENS		
Total cultures	Cultures showing growth	Percentages of cultures showing growth		Total cultures	Cultures showing growth	Percentages of cultures showing growth
			minutes			
30	30	100	1	36	36	100
30	30	100	2	120	11	9
46	42	91	3	120	1	1
46	32	70	4	36	0	0
46	22	48	5	36	0	0
46	13	28	6	36	0	0
46	14	30	7	36	0	0
46	6	13	8	35	0	0
34	3	9	9	24	0	0
26	0	0	10			
16	0	0	11			
12	0	0	12			

The times required to sterilize spores of *C. oedematiens* were about one third of the times required for complete killing of *C. botulinum A* spores under like experimental conditions. The results here reported on the heat resistance of *C. botulinum A* closely approximate the maximum values obtained by Esty and Meyer in 1922.

A check was made in a few experiments upon the relative resistance of moist and dried spores of *C. oedematiens* and *C. botulinum A*. To obtain dried spores, sterile swabs were dipped into spore-filled cultures of the organisms in question and then placed in sterile empty test tubes; these tubes were replugged and incubated at 37°C. for one or more days. They were then exposed to steam in parallel with swabs that had recently been dipped into cultures of the same total length of incubation. In every instance it was evident that dried spores were considerably less resistant to heat than were the moist spores,—a fact which has been noted by previous workers (Esty and Meyer, 1922).

TABLE 3

15 pounds saturated steam, 121°C.

C. BOTULINUM A			TIME OF EXPOSURE	C. OEDEMATIENS		
Total cultures	Cultures showing growth	Percentages of cultures showing growth		Total cultures	Cultures showing growth	Percentages of cultures showing growth
			minutes			
			½	24	2	8
35	32	91	1	36	0	0
18	13	72	1½	24	0	0
36	8	23	2	24	0	0
17	3	18	2½			
36	1	3	3			
18	1	5	3½			
28	0	0	4			
12	0	0	4½			
30	0	0	5			
12	0	0	6			
6	0	0	7			
6	0	0	8			
6	0	0	9			

Moist spores of locally isolated strains of *Clostridium welchii* and *Clostridium tetani* were also tested for their resistance to saturated steam in a few instances. With the strains employed

C. welchii was definitely less heat-resistant than *C. oedematiens* but *C. tetani* approximated the resistance of *C. oedematiens* very closely.

The remaining experiments concerned the effects of residual air in the autoclave. In work with saturated steam, the spore-covered swabs had been inserted vertically into the sterilizer, which meant that the test organisms were located approximately at the center level of the chamber. In the following experiments the swabs were inserted horizontally at six different levels ranging from near the top to near the bottom of the sterilizing chamber. The upper two levels approximated the upper thermocouple, the middle two the middle thermocouple and the lower two swabs corresponded to the level of the lower thermocouple. *C. oedematiens* was the test organism employed in these experiments.

Unsaturated steam is in no way as efficacious a sterilizing agent as saturated steam. This was definitely manifested in various ways which will be discussed separately.

A. Air in the autoclave lowers the internal temperature to a considerable extent. This lowering of temperature is an uneven phenomenon inasmuch as air is heavier than steam and tends to stratify beneath the steam.

Six experiments were performed with an autoclave pressure of 10 pounds and with approximately 25 per cent of air in the sterilizer. As mentioned above, the test swabs occupied six different levels and temperatures were measured by thermocouples at 3 different levels in the sterilizing chamber. In all these tests the three thermocouples in saturated steam indicated a temperature of 115°C. ±1°C., while the temperatures shown with 25 per cent air approximated 114°C. for the upper thermocouple, 113°C. for the middle and 108°C. for the lower thermocouple. The cultural results in the various tests were fairly uniform and the following protocol is considered as representative of this entire series of experiments.

Table 4 shows that 25 per cent air in the autoclave at a gauge pressure of 10 pounds caused the time required for sterilization of any swabs to be at least twice that of the controls in saturated steam; this differential between unsaturated and saturated steam was increased to at least five times when only the lowest swabs were taken into consideration.

Two experiments with 15 pounds pressure and 25 per cent air bore out the above findings. All control swabs were sterilized by 1 minute of exposure to 15 pounds of saturated steam whereas over 6 minutes was required to sterilize all swabs when 25 per cent air was present at this pressure.

B. A mixture of air and steam at any temperature is not as efficient a sterilizing agent as saturated steam at that same temperature. Four experiments bearing on this point were performed and the results of the different tests fell into close agreement. For unsaturated steam 20 pounds gauge pressure was employed and all the air possible (about 42 per cent) was left in the sterilizer. Swabs at different levels were placed in direct contact with this unsaturated vapor in the test runs. In control experiments saturated steam at 6 and 10 pounds gauge pressure was used. Table 5 shows the results of a representative experiment.

TABLE 4

Test organism—C. oedematiens

10 POUNDS STEAM WITH 25 PER CENT AIR IN AUTOCLAVE					CONTROLS 10 POUNDS SATURATED STEAM	
Thermocouple readings						
Upper 114°C. Middle 113°C. Lower 108°C.					Upper, middle and lower 115°C.	
Cultural results						
Total number of swabs	Number of swabs showing growth			Time of exposure	Total number of swabs	Number of swabs showing growth
	Lower	Middle	Upper			
				2 minutes	12	0
				3 minutes	12	0
12	4	4	4	4 minutes		
12	4	4	0	6 minutes		
12	4	0	0	8 minutes		
12	1	0	0	10 minutes		

Table 5 demonstrates two findings of particular interest:–(1) The temperature in the lower part of the autoclave was recorded as 115°C. both when 20 pounds gauge pressure of steam with 42 per cent air and when saturated steam at 10 pounds pressure were utilized for purposes of sterilization. In spite of this fact it took over four times as long (10 minutes) to sterilize the test swabs with the unsaturated vapor as was the case (2 minutes) with saturated steam. (2) The temperature recorded with 6 pounds of saturated steam was 110°C. Nevertheless the experiment indicates that 6 pounds of saturated steam in direct contact with the test swabs proved to have sterilizing properties which were as effective as those shown by unsaturated steam at 20 pounds pressure and possessing a minimum temperature of 115°C. These findings were borne out by the other experiments.

TABLE 5

Test organism—C. oedematiens

<table>
<tr><th colspan="5" rowspan="2">20 POUNDS STEAM WITH 42 PER CENT AIR IN AUTOCLAVE</th><th colspan="4">CONTROLS</th></tr>
<tr><th colspan="2">6 pounds saturated steam</th><th colspan="2">10 pounds saturated steam</th></tr>
<tr><th colspan="9">Thermocouple readings</th></tr>
<tr><td colspan="5">Upper 120°C.
Middle 119°C.
Lower 115°C.</td><td colspan="2">110°C.</td><td colspan="2">115°C.</td></tr>
<tr><th colspan="9">Cultural results</th></tr>
<tr><th rowspan="2">Total number of swabs</th><th colspan="3">Number of swabs showing growth</th><th rowspan="2">Time of exposure</th><th rowspan="2">Total number of swabs</th><th rowspan="2">Number of swabs showing growth</th><th rowspan="2">Total number of swabs</th><th rowspan="2">Number of swabs showing growth</th></tr>
<tr><th>Lower</th><th>Middle</th><th>Upper</th></tr>
<tr><td></td><td></td><td></td><td></td><td>2 minutes</td><td></td><td></td><td>12</td><td>0</td></tr>
<tr><td>12</td><td>4</td><td>0</td><td>0</td><td>4 minutes</td><td></td><td></td><td></td><td></td></tr>
<tr><td>12</td><td>4</td><td>0</td><td>0</td><td>6 minutes</td><td></td><td></td><td></td><td></td></tr>
<tr><td>12</td><td>4</td><td>1</td><td>0</td><td>8 minutes</td><td>12</td><td>2</td><td></td><td></td></tr>
<tr><td>12</td><td>0</td><td>0</td><td>0</td><td>10 minutes</td><td>12</td><td>0</td><td></td><td></td></tr>
</table>

C. Air in the autoclave cuts down the penetration of steam to a considerable extent. Two experiments were performed upon the rate of penetration of steam into uniform packages of rubber gloves. Each package consisted of a glove book containing two gloves. Two test swabs were thrust into the finger of each glove. A cloth wrapper was folded loosely around each package and tied with string. One thermocouple lead was inserted as closely as possible into the center of each package while a second lead ran to the outside. Only one package was employed in each test run and it was placed in the center of the sterilizing chamber. Fifteen pounds gauge pressure of steam with 25 per cent air was contrasted with 15 pounds of saturated steam.

Table 6 shows the results of one of these experiments. The thermocouple readings indicate that with unsaturated steam it took over 3 minutes for the inside of the loosely wrapped packages to come up to the temperature of the free chamber space of the autoclave, whereas with saturated steam these temperatures equalized themselves almost instantly. The cultural results show that sterilization was completed by saturated steam in 2 minutes while unsaturated steam required longer than 8 minutes to accomplish the same results.

TABLE 6

Experiment--Test organism, C. oedematiens

Air in the autoclave cuts down the penetration of steam to a considerable extent.

Uniform glove packets placed in autoclave. Each package contained 4 contaminated swabs. One thermocouple lead ran to the center of each package, a second lead being placed outside each package.

15 POUNDS STEAM WITH 25 PER CENT AIR IN AUTOCLAVE			CONTROLS: 15 POUNDS SATURATED STEAM IN AUTOCLAVE		
Exposure	Cultures	Growth	Exposure	Cultures	Growth
min.			*min.*		
5	4	+	2	4	–
8	1	+	3	4	–
	3	–			
11	4	–			
The inner thermocouple took from 3 to 4 minutes to come up to the outer thermocouple reading of 115°C.			The inner and outer thermocouples came to the same reading of 121°C. within one half minute.		

DISCUSSION

It has long been realized that saturated steam under a pressure of 10 pounds or more is an extremely efficient sterilizing agent.

Direct contact with 10 pounds of saturated steam will probably kill all surgically significant spores within 6 minutes and should even destroy the highly resistant spores of *Clostridium botulinum* within 20 minutes. When the pressure of saturated steam is increased to 15 pounds, complete sterilization should be obtained in approximately one third of the times mentioned above. In actual practice a margin of safety considerably above these times is customarily allowed for a number of reasons among which are the following:

1. It is at least conceivable that some spores may exist which are more heat-resistant than any yet reported.
2. Penetration of saturated steam into various packages or bundles may take considerable time even in a perfectly functioning autoclave.
3. Air is not always eliminated perfectly from the free chamber space in the average autoclave. Even when such elimination of air from the free chamber space has occurred, it often happens that local pockets of air may be trapped within the materials that are being sterilized. Local areas of unsaturated steam will result and sterilization in these local areas will be retarded.
4. Spores may become coated by various oily materials and thus be somewhat protected from free contact with saturated steam. The work of Dickson, Burke, Beck and Johnston (1925) appears to show that this is a point of considerable practical importance.

The experiments here reported indicate that the complete saturation of steam throughout an autoclave is a most important factor in the process of sterilization. If dilution with air takes place the efficiency of the sterilizing process is considerably reduced and this reduction is not merely a function of a reduction in temperature. It was clearly shown that a mixture of steam and air at a given temperature is a less efficient sterilizing agent than is saturated steam at that same temperature.

Our findings in this respect differ markedly from those recently published by Savage (1937). This author placed dry spores in dry glass tubes and inserted bits of cotton wool as sources of steam. These tubes were then evacuated to varying degrees, sealed and heated for definite intervals at varying temperatures in a glycerol bath. Cultures were subsequently made and the results showed that residual air in the tubes had no effect on the sterilizing process. One should note, however, that our experimental set-up approximates actual working conditions in an autoclave much more closely than was the case in the experiments which Savage reported.

SUMMARY AND CONCLUSIONS

Experiments were performed in an autoclave on various spore-forming anaerobic bacteria to compare the sterilizing effects of saturated steam with mixtures of air and steam.

The autoclave was constructed to permit extremely rapid entrance and discharge of steam, direct temperature readings could be made from three levels in the chamber and the air content of the chamber could be quickly determined. The chief organisms employed were *Clostridium oedematiens* and *Clostridium botulinum A*.

The following thermal death times were determined for direct contact with saturated steam:

	C. BOTULINUM A	C. OEDEMATIENS
	minutes	*minutes*
6 pounds saturated steam, 110°C.	Over 30	10
10 pounds saturated steam, 115°C.	10	4
15 pounds saturated steam, 121°C. ..	4	1

Dried spores were found to be less resistant than moist spores.

Air in the autoclave was shown to lower the efficiency of sterilization in the following ways:

A. Air in the autoclave lowers the internal temperature. This is an uneven phenomenon inasmuch as air is heavier than steam and tends to stratify beneath the steam.

B. A mixture of air and steam at any temperature is not as efficient a sterilizing agent as saturated steam at that same temperature.

C. Air in the autoclave cuts down the penetration of steam to a considerable extent.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTH CENTRAL BRANCH

UNIVERSITY OF MINNESOTA, MAY 20-21, 1938

CONJUNCTIVITIS. A STUDY OF THE ETIOLOGY OF 622 CONSECUTIVE CASES.[*] *J. H. Allen, and M. A. Wood, University of Iowa.*

During 1937, in the Eye Clinic at the State University of Iowa Hospitals, 622 consecutive cases of conjunctival inflammation were observed. Catarrhal conjunctivitis was the clinical diagnosis of 448 cases. *Staphylococcus* was the most frequent etiologic agent in this group with *Diplococcus pneumoniae* second, *Streptococcus* third and *Hemophilus influenzae* fourth.

The series included 55 cases of conjunctivitis of the newborn, none of which was caused by *Neisseria gonorrhoeae*. *Staphylococcus* again was the most frequent etiologic agent with the virus of inclusion blennorrhea second and *D. pneumoniae* third.

Lacrimal conjunctivitis was the diagnosis of 45 cases in which *D. pneumoniae* was most frequently found.

There were 39 cases of trachoma, 32 of which had a secondary bacterial infection. *D. pneumoniae* was the most frequent cause of the secondary infection in this group with *Staphylococcus* second and *H. influenzae* third.

The remaining cases included 21 of vernal conjunctivitis, 2 of phlyctenular conjunctivitis, 2 of chemical conjunctivitis, and 1 of ocular pemphigus.

SOME FACTORS AFFECTING THE BACTERIAL POPULATION OF FRESHWATER LAKES. *W. H. Stark, Janice Stadler, and Elizabeth McCoy,* University of Wisconsin.

It has been known for some time that the bacterial population of stored lake water is many times greater than that found in the lake itself. Increasing the area of solid surface per given volume of lake water was found by growth curve studies to increase the number of bacteria in stored water; this supports ZoBell's results with ocean water studies. To determine if accumulation of organic matter upon the surfaces might be the mechanism of surface effect a method was developed based upon oxidation of the organic matter with a sulfuric acid-potassium dichromate reagent. Measurable amounts of organic matter were found to accumulate over a period of hours on the surface of chemically clean glass slides. The accumulation was detected upon slides sterilized and suspended in sterile lake water, showing that accumulation is independent of and precedes bacterial growth. It would seem then that surfaces by accumulation provide a suitable concentration of nutrient material for greater bacterial development; and that although there is sufficient nutrient material in lake water for greater numbers of bacteria than are found naturally, the low concentration of this nutrient material is one of the important factors controlling the growth of bacteria in the open lake.

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[*] Part of a study being conducted under a grant from the John and Mary R. Markle Foundation.

STUDIES OF FRESHWATER BACTERIA: V. THE DISTRIBUTION OF SIDEROCAPSA IN SOME LAKES. *Yvette Hardman and A. T. Henrici*, University of Wisconsin.

GROWTH FACTORS AND NITROGEN FIXATION BY RHIZOBIUM TRIFOLII. *P. M. West and P. W. Wilson*, University of Wisconsin.

An attempt has been made to verify the recent claims of Verner and Kovaler that the root-nodule organism is capable of fixing atmospheric nitrogen in the presence of a concentrated "Bios" preparation, freed of a large part of its contaminating nitrogen. Although great stimulation was evident in growth of *Rhizobium trifolii* on the addition of extremely small amounts of "Bios" concentrates to a basal medium almost free of combined forms of nitrogen, analyses showed that no fixation had occurred.

Similar negative results were also obtained in the presence of Miller's Bios, Allison and Hoover's *Azotobacter* extract, crystalline vitamin B_1 and lactoflavin, with mannitol, oxalacetic, or succinic acids as sources of energy. The organism apparently, can make excellent growth without utilizing atmospheric nitrogen on as little as 0.001 mgm. of nitrogen per cc., if proper media are used for its cultivation.

MUTATION OF POLIOMYELITIC VIRUS INTO ENCEPHALITIC VIRUS. *Edward C. Rosenow*, Division of Experimental Bacteriology, The Mayo Foundation, Rochester, Minnesota.

In three experiments in which fourteen monkeys were inoculated intranasally with highly virulent poliomyelitic virus, mutation into encephalitic virus occurred in six monkeys; eight developed typical poliomyelitis. Three of the six monkeys in which mutation occurred had received the virus four days after six daily intranasal instillations of, and feeding of, poliomyelitic streptococcic vaccine in a concentrated sugar solution (partial immunity). One had received saline and concentrated sugar solution and two were vaccinated intranasally and orally with poliomyelitic streptococcic serum-vaccine after inoculation with virus (partial immunity). The encephalitic mutant virus proved virulent for mice, guinea-pigs, rabbits and monkeys, whereas the unchanged virus was effective as usual only in monkeys.

Feeding of a vaccine prepared from the streptococcus isolated from the mutant encephalitic virus protected mice in marked degree against intralingual injection of this virus. Feeding of a vaccine prepared with the streptococcus isolated from the unchanged virus had moderate protective action, while an arthritic streptococcic vaccine had no protective action. The streptococcus isolated from the encephalitic mutant virus was agglutinated to a greater degree and in higher dilution by the encephalitis antistreptococcic serum than by the poliomyelitis antistreptococcic serum, whereas in the case of the streptococcus isolated from the unchanged virus the reverse was true.

PROTECTION OF MONKEYS (MACACUS RHESUS) AGAINST EXPERIMENTAL POLIOMYELITIS WITH VACCINE AND ANTISERUM PREPARED WITH THE

STREPTOCOCCUS FROM POLIOMYELITIS.

Edward C. Rosenow, Division of Experimental Bacteriology, The Mayo Foundation, Rochester, Minnesota.

All of six monkeys that had received six daily intranasal instillations of, and that had been fed, poliomyelitic streptococcic serum-vaccine remained well after the first intranasal inoculation of virus given four days after completion of immunization. Two succumbed to poliomyelitis after the second and one succumbed after the third inoculation. Three resisted five inoculations of virus. Five of the six control monkeys succumbed to poliomyelitis after the first inoculation of virus. Of four monkeys vaccinated subcutaneously, two resisted the first inoculation, one succumbed to the third inoculation, while one resisted five inoculations of virus. Of four monkeys immunized intranasally and orally with arthritic streptococcic serum-vaccine, two succumbed to the first, one to the second, and one to the third, inoculation of virus.

Five intranasal inoculations (eight to thirty days apart) of virus treated for an hour and a half with poliomyelitic antistreptococcic serum (1:10) were without effect on four monkeys that had received, six hours previously, intranasal instillations of poliomyelitic antistreptococcus serum diluted 1 in 10. All of four monkeys treated in like manner, but in which the arthritic antistreptococcus serum was substituted for poliomyelitic antistreptococcus serum, succumbed to poliomyelitis, as did four control monkeys which received salt solution instead of antiserum.

FURTHER ATTEMPTS TO CONCENTRATE THE VIRUS OF POLIOMYELITIS.

W. C. White and Paul F. Clark, University of Wisconsin.

STUDIES ON THE NASAL PORTAL OF ENTRY OF POLIOMYELITIS VIRUS.

A. F. Rasmussen, Jr. and Paul F. Clark, University of Wisconsin.

AMINO ACID REQUIREMENTS OF THE LACTIC ACID BACTERIA.

A. A. Andersen, H. G. Wood and C. H. Werkman, Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa.

The addition of 19 purified amino acids, each in a concentration of 0.0075 per cent, to a basal medium of glucose 1 gram, $(NH_4)_2SO_4$ 0.3 gram, NaOAc 0.6 gram, inorganic salts, aneurin 1.0 gamma, riboflavin 50 gammas and ether extract of 3.0 grams yeast extract, per 100 cc. of medium, provided good growth of lactic acid bacteria (*Lactobacillus mannitopeous* L2, *L. buchneri* L4, and *L. lycopersici* L5). Acid production was used as a criterion of growth.

Tryptophane was found essential for *L. buchneri* but not for the other two. Cystine and threonine were essential for all three species, while serine and methionine were markedly stimulating if not essential.

By varying the concentration of one amino acid and maintaining the remaining 18 at 0.0075 per cent, it was found that approximately 75 gammas of threonine, 45 gammas of cystine and from 25 to 75 gammas of serine per 10 cc. of medium gave maximum acid production with L2 and L4.

A TECHNIQUE FOR STAINING AND COUNTING YEAST SPORES.

C. R. Arnold, Iowa State College.

A technique was developed which was particularly well suited for staining smears of yeast cultures in which the progress of sporulation was being followed by periodically counting the proportion of sporulated cells.

The staining procedure consisted in transferring a small quantity of the yeast culture to a glass slide where it was thoroughly mixed with a loopful of sterile, fresh skimmed milk which had previously been diluted with one part of sterile water. The smear was then fixed in 95 per cent alcohol for two minutes, dried, and stained with carbol fuchsin for two minutes at a temperature sufficient to make the staining solution steam. The smear was decolorized by dipping into a solution of 2 per cent acetic acid for an instant and then rinsing in 95 per cent alcohol. Finally, the smear was counterstained in aqueous methylene blue for 60 seconds.

Since there is maximum contrast between the sporulated (red dots) and non-sporulated cells, there is little difficulty in detecting the presence of spores even though they may be few and far between. The milk employed tends to break up lumps of cells at the time of mixing; later it prevents the cells from clumping when the smear dries, and finally it forms a blue background for the stained smear.

STAINING OF BACTERIAL FLAGELLA. *Einar Leifson*, Department of Bacteriology, University of South Dakota.

The technique of staining flagella published by the author in 1931 has been thoroughly re-investigated. A somewhat simpler, better, and more reliable stain has been perfected. The new stain has been prepared in the form of a dry powder which seems to be stable indefinitely. The staining solution is prepared by dissolving a definite quantity of the powder in a 35 per cent solution of alcohol. The solution is stable for a week or more. The solution is poured on the bacterial smear and the staining is complete in about 10 minutes. No counterstain is necessary.

The composition of the stain is as follows: Tannic acid 0.85 per cent, sodium chloride 0.5 per cent, pararosaniline acetate 0.35 per cent, alcohol (95 per cent) 35 per cent, water. The solid ingredients are dissolved in the alcoholic solution. Young (8-16 hour) agar slant cultures are suspended in distilled water to make a lightly turbid solution. A loopful of the suspension is allowed to run down a perfectly clean slide which has ¾ of its surface ringed around with a wax pencil to confine the stain. The stain is heaped up on the slide and allowed to act for about 10 minutes, then washed off.

Many interesting data have been obtained on the effect of the kind and concentration of electrolyte in the stain. Space does not permit of any detailed discussion but these data will be published elsewhere.

PRECIPITIN REACTION IN PNEUMONIA. *W. P. Larson and Milton Levine*, University of Minnesota.

Sera from thirty cases of lobar pneumonia, including types I, II, III, IV, VII, VIII, and XV, were tested against precipitinogens of types II, III, IV, and XXVII, using the precipitin ring test.

The precipitinogen was prepared by dissolving the residue from a liter of an 18-hour broth culture of pneumococci in 10 cc. of 0.5 per cent sodium ricinoleate and 0.5 per cent Ca and Mg free NaCl to give a final dilution of 1:10. This was further diluted to 1:100, 1:500, and 1:1000.

All the sera gave positive precipitin reactions in 15 minutes against the four precipitinogens tested. Controls taken from sera sent in for Wasserman tests were negative at the end of one hour, and then began to show a vague cloudy precipitate at the ring interface. This nonspecific reaction was due to the sodium ricinoleate since the soap alone gave the reaction against the control sera.

Seven type I sera gave an immediate and more pronounced precipitate against all the antigens tested.

The sera were drawn from two days after the onset of symptoms, up to two weeks in some cases.

DISSIMILATION OF CITRIC ACID BY AEROBACTER INDOLOGENES.

C. R. Brewer and C. H. Werkman, Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa.

The anaerobic dissimilation of citric acid by growing cells and non-proliferating cell suspensions of *Aerobacter indologenes* was qualitatively and quantitatively investigated. Principal products found were acetic, formic and succinic acids, carbon dioxide and hydrogen. Small amounts of acetylmethylcarbinol, 2,3-butylene glycol, ethyl alcohol and lactic acid were also reported.

The fermentation of citric acid by *Aerobacter indologenes* does not readily occur in media below pH 6.

Manometric investigations using the Warburg respirometer revealed that the anaerobic dissimilation of citrate was stopped by 0.003 M $NaAsO_2$, 0.1 per cent $NaHSO_3$ and 0.01 M $CH_2ICOONa$; 0.02 M NaF partially inhibited citrate fermentation; and 0.03 M malonate stimulated the production of gas from citrate as well as from fumarate and malate.

Aerobically, arsenite and iodoacetate also completely inhibited the dissimilation of citrate. NaF and 0.02 M $Na_4P_2O_7$ did not affect aerobic dissimilation. KCN (0.05 per cent and 0.1 per cent) partially inhibited.

The evidence was examined from the viewpoint of the citric acid cycle of Krebs and Johnson and it was concluded that the cycle does not apply to the bacterial dissimilation.

BEHAVIOR OF ADDED HYDROGEN ACCEPTORS ON THE METABOLISM OF AEROBACTER INDOLOGENES.

M. N. Mickelson, H. Reynolds and C. H. Werkman, Department of Bacteriology, Iowa State College, Ames, Iowa.

Acetaldehyde, propionaldehyde, butyraldehyde, and acetic, propionic and butyric acids were added to glucose fermentations by *Aerobacter indologenes* to observe their effect on the production of 2,3-butylene glycol and acetylmethylcarbinol. In view of the pronounced effect of pH on the dissimilation by these organisms, acetaldehyde and acetic acid were added under acid and alkaline conditions. Under acid conditions, added acetic acid disappears and is quantitatively accounted for as 2,3-butylene glycol. Under alkaline conditions added acetic acid is not attacked and acetic and formic acids accumulate. Acetaldehyde added to an alkaline fermentation (above pH 7.0) quantitatively is converted into ethyl alcohol and acetic acid in equimolar quantities, in an acid fermentation (below 6.2) acetaldehyde causes increases in ethyl alcohol, acetic acid, acetylmethylcarbinol and 2,3-butylene glycol.

When propionic acid is added to an acid fermentation, it is reduced to propyl alcohol, there is a reduction in yield of ethyl alcohol and a corresponding increase in butylene glycol and acetylmethylcarbinol. Butyric acid under similar conditions is not attacked.

Propionaldehyde and butyraldehyde were recovered as the corresponding acids and alcohols with no increase in 4-carbon neutral compounds.

INFLUENCE OF METHODS OF HANDLING THERMODURIC LACTICS ON THE HEAT RESISTANCE OF SUBCULTURES.

H. J. Peppler and W. C. Frazier, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

Cultures of *Lactobacillus helveticus* (39aW) and *Streptococcus thermophilus* (C_3) were carried separately in reconstituted skim milk at 37°C. and also at 37°C. plus different periods at 20°C. The cultures grown continuously at 37°C. were transferred to fresh reconstituted skim milk every 12 and every 24 hours. Additional series of cultures were incubated at 37°C. for 12 hours followed by 36, 84, and 154 hours at 20°C., and for 24 hours at 37°C. followed by periods of 24, 72, and 144 hours at 20°C.

After each culture in each series had been transferred to fresh medium at least seven consecutive times, a definite amount was inoculated into a large flask of fresh medium, heated for 30 minutes at 60°C. and incubated at 37°C. The rate and amount of growth and the degree of acid development were determined.

Cultures of *L. helveticus* which were carried continuously at 37°C., either in ordinary medium or in one containing 0.1 per cent added peptone, were equal in growth and acid production following the heat treatment. *S. thermophilus* transferred every 12 hours at 37°C. increased in numbers and developed acid more rapidly after the heat treatment than cultures transferred every 24 hours.

When each organism was grown at 37°C. for 12 hours followed by incubation at 20°C. for periods longer than 36 hours, a significant decrease in activity was observed.

These results suggest information which may be applicable under certain conditions arising in the Swiss cheese plant.

THE BUTYL ALCOHOL FERMENTATION OF WOOD SUGAR.

N. O. Sjolander and A. F. Langlykke, University of Wisconsin.

Sugars obtained from wood by hydrolysis with dilute sulphuric acid at elevated temperatures were readily fermented by the butyl anaerobes, *Clostridium felsineum* and *Clostridium butylicum*. The hydrolyzates were prepared for fermentation by treatment with calcium carbonate, lime and Norite. A malt-sprouts medium was most satisfactory for maximum wood-sugar utilization. The addition of corn and other supplementary carbohydrate was unnecessary.

Whereas yeasts were capable of fermenting only the hexoses in wood sugar, *C. felsineum* and *C. butylicum* were able to ferment the pentoses also. Wood sugar in concentrations up to about 5 per cent was almost completely fermented. From 30 to 40 per cent of the fermented sugar was converted into neutral volatile products. The distribution of products formed by *C. felsineum* was practically the same from wood sugar as from glucose. Butyl alcohol was the principal neutral volatile product; smaller amounts of ethyl alcohol and acetone were formed. *C. butylicum* produced, in addition, considerable amounts of isopropyl alcohol—more from wood sugar than from glucose. It was shown that this additional isopropyl alcohol arose from the acetic acid present in the wood hydrolyzates.

DESTRUCTION OF BACTERIA BY CERTAIN FISH LIVER OILS.

Gordon Worley and Horace R. Getz, University of Wisconsin.

Cod Liver Oil Treatment of Experimental Tuberculous Skin Ulcers.

Theodore A. Koerner and Horace R. Getz, Department of Medical Bacteriology, University of Wisconsin.

Reports of the favorable response of a few cases of lupus vulgaris to topical application of whole native cod-liver oil have led to an experimental study of the healing action of cod-liver oil for cutaneous tuberculosis.

Guinea pigs, uniformly free from spontaneous tuberculosis as shown by tuberculin testing with 10 mgm. OT, and weighing 500 grams or more, developed 5-15 mm. ulcers in 3-4 weeks following the intradermal injection of 0.2 mgm. of virulent human tubercle bacilli, strain H 37 in 3 cc. saline, on the nape of the neck. Several hundred animals kept on the same ration throughout were used in the course of 8 series of experiments.

In the course of the work a total of 86 animals received applications of whole cod-liver oil twice daily between the 28th and 40th days of infection. At this time 60 per cent of the lesions were not only shut but healed underneath. A total of 89 control animals showed 40 per cent closing of lesions by epithelialization during the same period but the closing in a goodly number of cases was only temporary and poor. Necrotic undermining foci were found beneath the epithelium.

Similar numbers of animals were used in tracing the healing activity of the oil into a chemical fraction designated as the "Vitamin Fraction," comprising 0.3 per cent of the whole oil, and containing its vitamin A and D stores. Experiments, as yet inadequate, suggest that neither of these agents or their combination are responsible for the healing activity of the oil.

Vitamin A Deficiency of Normal and Tuberculous Individuals as Indicated by the Biophotometer.

Horace R. Getz and Milton Finn, University of Wisconsin.

H_2/CO_2 Ratios of Escherichia-Aerobacter.

M. Silverman and C. H. Werkman, Department of Bacteriology, Iowa State College, Ames, Iowa.

Change in pH markedly affects the H_2/CO_2 ratios of *Aerobacter indologenes*. Increase in pH from an acid to alkaline range causes a progressive change in the H_2/CO_2 ratio from 0.5 at a pH of 5.8 to 3.4 at an initial pH of 7.7. The gas ratio of *Escherichia coli* remains constant throughout this range. Added acetates and propionates reduced the H_2 evolved by *A. indologenes* by acting as hydrogen acceptors. Butyrates had no effect. Neither acetates, propionates, nor butyrates in any way affected the gas ratio of *E. coli*. Non-proliferating cells and the Warburg respirometer were employed in the above work.

A glucose-acetate medium for differentiation of members of the colon-aerogenes group was described.

The Antagonistic Action of Bacillus Thermophilus, Bacillus Subtilis, Escherichia Coli-Communis, and Alcaligenes Fecalis on Sarcina Lutea.

Olga Smith, Lawrence College, Appleton, Wisconsin.

Suspected antagonistic effect of *Bacillus thermophilus*, *Bacillus subtilis*, *Escherichia coli-communis*, and *Alcaligenes fecalis* on *Sarcina lutea* was investigated. Suspensions were made using *Sarcina lutea* together with one of each of the four organisms and nutrient agar plates were poured immediately upon mixing, after two hours incubation, and again after twenty-four hours incubation. To discover if antagonistic action would occur when colonies were planted directly from pure culture to a solid medium, *Sarcina* was plated to agar in pure culture with *B. thermophilus* and with *B. subtilis* without having been previously in mixed suspensions. An effort is also being made to ascertain the relative effect of the organisms in culture and of the filtrate of the antagonistic organisms.

The results indicate that:

(1) Antagonistic action was most evident in the *B. thermophilus*—*Sarcina* mixture.

(2) *B. subtilis* was only slightly less antagonistic toward *Sarcina* than *B. thermophilus*.

(3) *Escherichia coli-communis* and *Alcaligenes fecalis* were less antagonistic but even these organisms showed strong suppression of *Sarcina*.

(4) In the case of all four organisms, prolonged contact in broth resulted in greater suppression of *Sarcina*.

(5) *B. thermophilus* and *B. subtilis* were not only active in inhibiting *Sarcina* in mixed broth cultures, but possessed the ability to inhibit its growth when planted to agar in pure culture.

(6) The filtrate of *B. subtilis* does not suppress *Sarcina* to as great an extent as the live cultures.

Further work on the action of the filtrate of the antagonistic organisms is now in progress.

A CASE OF ACUTE BACTERIAL ENDOCARDITIS CAUSED BY ESCHERICHIA COLI. *W. H. Stevens and J. L. Parks*, University of Wisconsin Medical School.

A patient (A. T.) at the Wisconsin General Hospital with a diagnosis of acute endocarditis gave blood and urine cultures positive for *Escherichia coli*. Progressive cardiac failure was accompanied by chills, fever, anemia, and leucocytosis, and the patient expired one month after admission.

At post-mortem, obstructive vegetations were found in the aortic heart valve. Microscopically these showed abundant clumps of bacilli within the tissue; direct smear of this material yielded only gram-negative rods. This organism was isolated both from the vegetations and from the blood; when tested biochemically, culturally, and immunologically, it fell into the class *Escherichia coli*, variety *acidi-lactici*.

The probable source of infection was the right kidney, which showed multiple abscesses on post-mortem examination.

Of the cases of this type previously reported in the literature, only five are considered authentic according to the postulates proposed by Dickar, which require that the organism be present in histological sections of the heart valve as well as in the direct bacteriological smears. This case is shown to satisfy these criteria and is offered as an authentic one to be recorded.

EFFECT OF ENVIRONMENT ON NITROGEN EXCRETION BY LEGUMINOUS PLANTS. *Orville Wyss and P. W. Wilson, University of Wisconsin.*

Excretion of nitrogen by inoculated pea plants varied with the season in experiments made each month throughout the year. The most frequent occurrence of benefit to a non-legume in a pea-oat or a pea-barley mixture was noted when the growing period coincided with cool, long days and especially with sunlight of relatively low intensity but of long duration. This effect of the environment received confirmation by a greenhouse experiment in which three different temperatures were used with a long and a short day. Examination of the occurrence of positive and negative findings reported at other experiment stations indicate that excretion is more likely to obtain in those regions where the specified climatic conditions hold. The effect does not appear to arise from cool, long days *per se*, but such an environment usually results in a type of growth (high nitrogen plant) which favors the occurrence of excretion. The same type of growth brought about by other means, e.g., shading, likewise results in excretion.

DIRECT DETERMINATION OF FREE NITROGEN UPTAKE BY GASOMETRIC METHODS. *Charles Hurwitz and P. W. Wilson*, University of Wisconsin.

The Kjeldahl method for total nitrogen is subject to certain inaccuracies which render it unreliable as a technique for unequivocal demonstration of free nitrogen uptake by substrates high in total nitrogen, e.g., seeds, nodules, plant extracts. Changes in the forms of organic nitrogen which take place during the period of the experiment may give rise to errors in the determination which invalidate the apparent gains in total nitrogen observed. It is proposed that a direct measure of free nitrogen uptake be a necessary condition for acceptance of claims of nitrogen fixation by substrates initially high in nitrogen. A suitable method for direct demonstration of free nitrogen uptake has been developed. The substrate is placed in a 150 ml. Erlenmeyer flask which contains a short test-tube holding alkali for absorption of liberated CO_2. The flask is connected with a Novy-Soule respirometer which measures the oxygen uptake. As soon as the pressure of oxygen in the system is reduced to about 0.15 atm. through respiration, pure oxygen is added to bring it back to atmospheric pressure (0.21 atm.). Suitable precautions are taken to insure that no air enters the apparatus during addition of the oxygen. At the end of the experiment oxygen is added until the original pressure has been restored, then samples of the atmosphere in the system are taken for gas analysis. When tested with *Azotobacter*, the method checks quite satisfactorily with those obtained by Kjeldahl analyses. A sensitivity of less than one milligram of nitrogen is readily obtained.

COMPARATIVE RESULTS OBTAINED BY THE ELECTROMETRIC AND COLORIMETRIC METHODS OF DETERMINING BACTERIAL FERMENTATION. *G. M. Savage*, University of Minnesota.

It was suggested that some variability in fermentations might be attributable to the pH indicator used. Evidence consisted of a study of acid fuchsin (Andrade's indicator).

Shigella dysenteriae (Shiga type) was found to ferment only glucose and maltose when 0.002 per cent indicator was used; it fermented glucose, maltose, sucrose and lactose when 0.02 per cent indicator was used. The pH decrease, measured with a glass electrode, was the same for a given sugar, independent of the indicator concentration. None of the 0.02 per cent series became as acid as its corresponding sugar in the 0.002 per cent series, and yet this series resembled the *S. sonnei* and *S. dispar* fermentations.

In addition the color of single strength beef broth was found sufficient to mask the red color of 0.002 per cent indicator in buffer solutions, so that a tube of broth could drop from its initial pH of 7.6 to one of 6.3 without any fermentation being apparent. All such inapparent fermentations had to be observed electrometrically except those of the Shiga organism, which could be made apparent by adding more indicator.

Acid fuchsin was found not to exhibit a perceptible protein error in beef broth over the pH range of 5.0 to 7.5.

NORTH CENTRAL BRANCH

University of Wisconsin, Madison, Oct. 21, 1938

Studies on the Occurrence and Importance of Bacteria in the Sea. *Claude E. ZoBell,*[*] Scripps Institution of Oceanography, La Jolla, Calif.

Bacteria are found to be widely distributed in the sea although in numbers ranging from less than one to only a few hundred per cc. Their seasonal and vertical distribution is more closely correlated with the abundance of phytoplankton than with temperature, insolation, hydrostatic pressure or dissolved nutrients. The paucity of bacteria in sea water is attributed primarily to the lack of surface-active solids such as particulate matter, which seem to be requisite to the multiplication of bacteria in dilute nutrient solutions. Marine bottom deposits contain from hundreds to millions of bacteria per gram, the number of aerobes as well as anaerobes decreasing with core depth.

Nearly a hundred new and undescribed species of bacteria, which upon primary isolation grow in nutrient sea water media but not in corresponding freshwater media, have been characterized. The majority of them belong to the family *Bacteriaceae* with several other families and numerous genera represented. Many marine bacteria are extremely heat-sensitive, some failing to multiply after short exposures to temperatures no higher than 30°C. Although most of the marine bacteria are active at 0°C., their optimum temperature for multiplication is between 15 and 25°C., including those which have come from the deep sea bottom where the temperature is perpetually colder than 3°C.

The ways in which bacteria may influence chemical, physico-chemical, geological and biological conditions in the sea were discussed. It was also pointed out that marine bacteria have considerable economic importance.

A Preliminary Report on the Numbers and Distribution of Microorganisms in Beach Sands. *Nelson E. Rodgers and Newell O. Sjolander,* Department of Agricultural Bacteriology, University of Wisconsin.

Observations have been made on the numbers and distribution of microörganisms in the beach sands of four lakes of northern Wisconsin. Counts of the numbers of bacteria and protozoa in sand samples three centimeters deep taken at various distances from the water's edge have revealed a characteristic distribution. Near the water's edge plate counts of a few hundred thousand bacteria per cc. of sand and direct counts of a few hundred protozoa per cc. of sand are commonly observed. These numbers gradually increase up to a distance of between one and two meters from the water's edge. At this point the numbers of both protozoa and bacteria show a sharp rise. In this active region two to eight million bacteria per cc. of sand and ten to forty-five thousand protozoa per cc. of sand are commonly encountered. Beyond this densely populated region and up to the outermost edge of the beach the numbers of microörganisms usually decrease.

Observations on the vertical distribution of microörganisms in the most active region of the beach have demonstrated that, in a sand core six centimeters deep, 60 to 70% of the bacteria and 70 to 80% of the protozoa inhabit the top centimeter. Below the surface centimeter the numbers fall rapidly with increasing depth.

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[*] Now on sabbatical leave at the University of Wisconsin.

THE PEPTIDASES OF BACTERIA. *Julius Berger*, University of Wisconsin.

A KINETIC METHOD FOR THE STUDY OF BACTERIAL DEHYDROGENASES. *R. K. Tam and P. W. Wilson*, University of Wisconsin.

Through the use of appropriate light filters the Evelyn photometer can be used as a rapid and convenient method for the study of the kinetics of bacterial dehydrogenases. Special Thunberg tubes of uniform curvature and homogenous glass are prime requisites in the elimination of large experimental and correction errors.

Experiments with "resting cell" suspensions of the root-nodule organism show that a light filter in the region of 5,400 Å will give a straight-line relationship between methylene-blue concentration and the logarithm of the galvanometer readings. Filters above and below this region do not successfully eliminate interfering factors from the measurement of methylene-blue concentration.

Studies on the effect of hydrogen-ion concentration on glucose dehydrogenase reveal a constant rate of dye reduction in the acid range while in the alkaline range an acceleration inevitably results after reduction has proceeded for a few minutes. The formation of intermediate oxidation products and the adaptation of the enzyme to unfavorable alkaline conditions are factors involved. Further kinetic studies with the root-nodule bacteria will include the effect of various substrates and inhibitors.

CHROMOSOME NUMBERS IN ROOT NODULES AND ROOT TIPS OF CERTAIN LEGUMINOSAE. *Louise Wipf*, University of Wisconsin.

The tetraploid chromosome number was found in infected cells of root nodules of 31 diploid plants including 6 species from the genus *Medicago*, 11 species of *Melilotus*, 9 species of *Trifolium*, 3 species of *Lathyrus*, and one each from *Pisum* and *Vicia*. In all these, the uninfected cells of the nodular cortex possess the somatic (diploid) number of chromosomes typical of the host species.

The chromosome number in infected nodular cells in diploid and autotetraploid strains of *Melilotus alba* Desr. is twice that found in uninfected somatic cells of the respective plants. The chromosome number in infected cells of the nodules from diploid, triploid, tetraploid, and octoploid forms of the genus *Medicago* is likewise, double that in the root tip cells. Infected nodular cells from plants of a natural polyploid series in the genus *Trifolium* possess twice the normal somatic number of chromosomes.

Inhibition of the fixation of free nitrogen by such factors as a "poor" strain of *Rhizobium*, the presence of combined nitrogen in the nutrient, of the presence of hydrogen or carbon monoxide gas does not affect the number of chromosomes in the nodular cells. Infected cells in every case possess twice the somatic number found in uninfected cells.

There is definitely and consistently a two-to-one chromosome ratio between infected and uninfected cells of the root nodules of the leguminous plants examined.

THE DESTINATION OF NITROGEN FIXED WITHIN LEGUMINOUS NODULES. *G. Bond*, University of Wisconsin and University of Glasgow, Scotland.

The initial destination of nitrogen fixed within legume nodules appears to depend on certain environmental conditions. Under many growth conditions the whole of the fixed nitrogen is retained within the plant-nodule system. Under certain circumstances there may be an excretion of a very substantial proportion of the fixed nitrogen into the rooting medium, as established by the experiments of Virtanen. Other workers, including the present writer, have been unable to secure excretion with the same facility as Virtanen, if at all. Examination of Virtanen's results shows that his conditions produce a very high rate of fixation relative to the growth of the legume, so that an excess of nitrogen will tend to arise within the nodules. Reports from various stations make it questionable whether normal growth conditions do regularly produce the requisite high relative rate of fixation, so that the precise significance of nodule excretion in agricultural practice has yet to be established.

SPECIFICITY STUDIES IN THE SO-CALLED "E" GROUP OF LEGUMINOUS PLANTS. *J. C. Burton and L. W. Erdman*, The Nitragin Company, Inc., Milwaukee, Wisconsin.

Because of the large number of leguminous crops of agricultural importance now in the "E" or Cowpea cross-inoculation group, and the diversity of this group both with respect to types of plants and reciprocal effective cross-inoculation between them, studies were begun in an attempt to find a logical, practical subdivision of this group from the standpoint of commercial production of inoculations for these legumes.

Preliminary studies were conducted to test the Rhizobia for efficiency in nitrogen fixation on their mother host-plant. Several of these strains were then tested for efficiency on the velvet bean and three varieties of peanuts. Strains efficient on one host plant in this group were not necessarily efficient on other members of the group. Few of the strains from other members of the group were efficient on peanut. The indications were that such a division of the cowpea group would be very beneficial in supplying the farmers with a more efficient inoculation for these legumes.

THE HEAT RESISTANCE OF LACTIC ACID BACTERIA GROWN WITH MYCODERMA. *H. J. Peppler and W. C. Frazier*, University of Wisconsin.

It has been observed with certain cultures of thermoduric lactic acid bacteria grown in association with *Mycoderma*, that the bacteria present in the vicinity of the mycoderm film are more heat resistant than the bacteria located at some distance from the pellicle.

When cultures of *Streptococcus thermophilus*, strain Mc-my, were grown in reconstituted skim milk at 37°C. for 14 hours and then stored at 20 or 25°C. for one week, subcultures of the top, or the mycoderm zone, showed greater rates of growth and of acid development at 37°, after heat treatments at 64° for 30 minutes, than exhibited by the subcultures of the bottom regions of the same parent cultures. The effect of the mycoderm was greatest in cultures stored at 25°. The mycoderm influenced the bacteria to a considerable depth, for the rates of growth and of acid production of heat-treated subcultures of the mixture of top and bottom were in between those of the subcultures of the top and of the bottom regions.

The association of the mycoderm with *Lactobacillus helveticus*, strain 39 a-my, at storage temperatures of 20 or 25°C. did not result in significantly greater heat resistance of the bacteria in the vicinity of the mycoderm film. Cultures grown for one week at 25°C., however, yielded subcultures from the mycoderm zone which were much more active after a heat treatment of 62° for 30 minutes than the subcultures of the bottom region.

THE LACK OF ONE OF THE SOMATIC ANTIGENS OF TYPHOID CULTURES. *Lois Almon and W. D. Stovall*, State Laboratory of Hygiene and University of Wisconsin.

Two out of one hundred and seventy cultures of *Eberthella typhosa* were found by studies of their reaction to a number of strains of bacteriophage to differ from the remainder of the group. Agglutinin absorption studies indicated that the difference lay in the lack of one of the heat-stable antigenic fractions.

In an effort to ascertain the identity of the missing antigen, cross agglutination and absorption studies were performed with normal typhoid cultures and with cultures of *Salmonella schottmülleri* as well as the two deficient cultures. No good evidence was obtained for the identity of the missing antigen with that somatic constituent which is common to *Eberthella typhosa* and *S. schottmülleri*, namely antigen XII of the Kauffmann scheme. Further absorption studies using *Salmonella enteritidis* culture and antiserum demonstrated that the fraction lacking in the deficient typhoid cultures was present in *S. enteritidis*.

It is tentatively concluded, therefore, that antigen IX of the Kauffmann scheme is not present in demonstrable amounts in the two typhoid cultures studied.

CENTRAL NEW YORK AND CENTRAL PENNSYLVANIA BRANCHES

JOINT MEETING, STATE COLLEGE, PA., MAY 21, 1938

SOME CULTURAL CHARACTERISTICS OF THE GENUS CORYNEBACTERIUM. *R. F. Brooks*, New York State Agricultural Experiment Station, Geneva, New York.

A progress report was given on studies of 24 diphtheroids isolated from fresh, normal milk. These cultures fell into two groups—one leaving litmus milk unchanged, the other giving a slightly alkaline reaction. Most of the first group did not reduce nitrates, while the second did. None of the cultures liquefied gelatin, produced indol, or hydrolysed starch. Further work is in progress on these cultures.

Of sixteen authentic strains of *Corynebacterium diphtheriae*, only one, Park-Williams No. 8, agrees entirely with accepted descriptions. Only two strains fail to reduce nitrates. Indol is not formed by any strain. Nine strains leave litmus milk unchanged. Nine strains form pellicle and sediment in nutrient broth. The strains are facultative aerobic. It is concluded that cultural characters cannot be relied upon for diagnosis of *C. diphtheriae*, animal inoculations being necessary.

THE OCCURRENCE OF YEAST CELLS IN A CARDIAC VEGETATION. *Arthur S. Brumbaugh*, 1312 Eleventh Street, Altoona, Pa.

Yeast cells are occasionally found in the pharyngeal exudate in tonsillitis or in the sputum from occasional cases of bronchitis and pneumonia. Mitchell (Jour. A.M.A. v. *106* #6) reports a case of meningeal torulosis. Mallory (New Engl. Jour. Med. June 14″ 1934) reports five cases of torulosis simulating Hodgkins Disease. Systemi Torulosis is however rather rare and the finding of yeast cells in a heart-valve vegetation was therefore thought to merit at least casual consideration.

Mrs. K. R., white, age 30 yrs. died at the Mercy Hospital, Altoona, Pa. Jan. 16″ 1935 of endocarditis and at the post mortem examination she was found to have general anasarca, enlarged spleen and upon examining the heart there was marked ulceration of the aortic cusps and a large vegetation practically blocked the aortic orifice. Upon sectioning this vegetation and staining by the Gram-Weigert method myriads of staphylococci and, in a certain portion of the sections, yeast cells, were found.

CULTURAL CHARACTERS OF CERTAIN FOOD-POISONING MICROCOCCI. *W. C. Haynes*, New York State Agricultural Experiment Station, Geneva, New York.

A substance elaborated by a yellow hemolytic micrococcus was established by Dack, Cary, Woolpert and Wiggers as the cause of a food poisoning outbreak in 1929. Since then micrococci of both the aureus and albus types have been incriminated in similar outbreaks. The Dolman kitten inoculation test is still the only reliable test for enterotoxin-producing ability of micrococci.

Enterotoxin-producing micrococci, in common with other micrococci are reported to be: (a) either white, yellow or orange; (b) hemolytic (rabbit erythrocytes); (c) generally gelatin liquefiers; (d) usually nitrate reducers; (e) fermenters of glucose, maltose, lactose, sucrose, mannitol but not of rhamnose; (f) capable of coagulating human and rabbit plasma.

Preliminary results of the present investigation indicate that they are also: (g) catalase positive; (h) unable to hydrolyze starch; (i) usually able to produce hydrogen sulfide; (j) unable to peptonize coagulated blood serum.

Using the classification of Hucker, it is evident that most enterotoxin-producing strains of micrococci are *Micrococcus aureus*, but that other strains have been reported which would be classed as *Micrococcus albus*, *Micrococcus candidus* or *Micrococcus aurantiacus*.

A GRAPHIC DIFFERENTIATION BETWEEN SECRETED AND ENDO-ENZYMES IN CULTURES. *Otto Rahn, A. D. Console, and R. E. Deuel*, Laboratory of Bacteriology, Cornell University.

If the enzyme remains in the cell, the rate of fermentation reaches its maximum when the number of cells is at the maximum. The point of inflection of the products curve can never occur later than the cell maximum. When the enzyme is secreted, secretion continues after the maximal cell number has been reached. The rate of product formation continues to increase, and the point of inflection is considerably later than the growth maximum.

As examples for endo-enzymes, lactic and urea fermentation were shown, as examples for secreted enzymes, gelatin decomposition by *Bacillus subtilis* and fat-splitting by *Pseudomonas fluorescens*.

THE MICROFLORA OF FLUE-CURED TOBACCO. *R. G. Harris, D. E. Haley, and J. J. Reid*, Division of Bacteri-

ology and Dept. of Agricultural Chemistry, The Pennsylvania State College.

Investigation of the microflora of flue-cured tobacco of the Virginia bright leaf type has revealed a marked influence of fertilizer treatment upon the types of organisms developing upon the cured leaf.

Leaves low in potassium and high in nitrogen have been found to be characterized by the development of Gram-negative rods. These organisms produce a yellow pigment on nutrient agar and the growth is dry and wrinkled. Leaves high in potassium and low in nitrogen, on the other hand, have been found to favor the development of Gram-negative rods, the growth of which on nutrient agar is smooth and moist. Both forms are found in almost equal numbers on flue-cured tobacco of normal potassium and nitrogen content. In addition, mycoderms have been found to develop on such tobacco. These organisms ferment carbohydrates slowly, decompose urea rapidly, and are able to utilize ethyl alcohol as an energy source.

THE ORGANISMS CAUSING RUSTY SPOT IN CHEDDAR CHEESE. *Robert S. Breed and Carl S. Peterson*, New York State Agricultural Experiment Station, Geneva, New York.

For some time bacteriologists have been interested in bacteria that develop during ripening, as rusty-colored colonies in the cracks of cheddar cheese. This defect apparently occurs in cheese from particular dairy farms in the cheddar district of England, or factories in America. The organisms were identified in 1897 and named *Bacillus rudensis* by Prof. Connell of Kingston, Canada. The name is derived from the first syllable of the name of his associate, Mr. Ruddick, later Dairy Commissioner of Canada.

The original cultures and later isolations at the Station were lost. Biochemical studies of re-isolations secured from material from Somerset County in England in 1926 and from the Dairy Research Institute at Reading, England have shown that these rusty-colored cultures belong to two species. Some are chromogenic variants of *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.*, which it is proposed to name *var. rudensis*. Others are variants of *Lactobacillus brevis* (Orla-Jensen) Bergey *et al.* and may also be named *var. rudcnsis*. These organisms show the typical characteristics of the species named, except that in shake cultures of potato broth gelatin they slowly develop a rusty-colored chromogenesis.

Other species of lactobacilli, streptococci and propionic acid bacteria may develop an orange to rusty-red chromogenesis.

A DISTRIBUTION STUDY OF CHROMOBACTERIUM LIVIDUM. *John W. Rice*, Bacteriological Laboratory, Bucknell University.

Because of the importance of the organism in question in a municipal water supply, bacteriological surveys were made on White Deer Creek in June of the years 1931, 1934 and 1936. There are 54 hunting camps, but no permanent human habitations located on the water shed.

Water samples were collected from mid-stream at mile intervals and from each tributary near its junction with the creek. These samples were tested promptly in a field laboratory in accordance with approved methods of the A.P.H.A. In a grand total colony count of 18,544 from 114 water samples tested in 1931, no dark purple colonies were observed on any of the culture plates; in 1934 a total colony count of 10,271 from 122 samples yielded one such colony; but in 1936 out of a total colony count of 33,128, seven deep purplish colonies were isolated. Subsequent laboratory studies revealed these microorganisms to be *Chromobacterium lividum* Voges, as described in Bergey's *Manual of Descriptive Bacteriology,* with the single exception that our cultures reduced nitrates. *Chromobacterium lividum* is apparently relatively rare in this drainage area, but is definitely on the increase. This increase may be, as yet obscurely, due to significant changes on the drainage basin: In 1931 the waters were extensively stagnated by 124 beaver dams; by 1934 these dams had all been removed, but 20 miles of new forest roads were under construction by the CCC in and across the drainage basin.

ALLERGIC SKIN TESTS FOR BRUCELLA INFECTION, *D. R. Cordy,* N. Y. State Veterinary College, Cornell University, Ithaca, New York.

A heat-killed *Brucella* saline suspension, a *Brucella* broth-culture filtrate, and a solution of *Brucella* nucleoprotein in saline 1:500, were used in 336 intradermal initial tests and retests of 56 *Brucella* infected guinea pigs and 32 uninfected controls. None of the artificially infected animals showed negative reactions, and none of the controls gave positive reactions. The broth culture filtrate showed 45 per cent doubtful reactions among infected, and 20 per cent doubtful reactions among control animals. The nucleoprotein elicited 19 per cent doubtful reactions among infected, and 4 per cent among control animals. Heat-killed suspensions resulted in 9 per cent doubtful reactions among infected, and 15 per cent among control animals. This latter agent gave equal results in densities of one billion and two billion organisms per cubic centimeter. Initial tests were successful by 9–15 days after infection, and retests were successful 17 days after a prior test. The skin test showed very close agreement with the agglutination test. Infection with a strain of low virulence did not modify skin sensitivity when compared with agglutination. Injection of dead *Brucella* did not produce skin sensitivity, nor did repeated skin testing sensitize uninfected animals. Tuberculin, dead *Escherichia coli,* broth, and physiologic saline rarely produced dubious reactions when inoculated intradermally.

BRUCELLA AGGLUTININS IN HORSES. *W. S. Stone,* N. Y. State Veterinary College at Cornell University, Ithaca, N. Y.

The incidence of *Brucella* agglutinins has been determined in three different classes of horses. Of 1172 samples obtained in New York City, 702 (64.6%) were completely negative in all dilutions and 113 (9.6%) reacted at 1:50 or higher. The highest titre obtained was agglutination at a dilution of 1:400.

205 samples have been obtained from country horses of which 110 (53.6%) were negative while 49 (23.0%) reacted at a dilution of 1:50 or higher. The maximum titre obtained was an agglutination at 1:6400.

Of 135 samples from clinical horses 59 (43.7%) were negative in all dilutions and 61 (45.2%) reacted at 1:50 or higher. There were 6 animals in this group that reacted at 1:6400, the highest titre obtained.

It should be borne in mind that the mere presence of agglutinins for the Bang organism in the blood of equines is not definite evidence that active disease is present in these animals. Very high titres for *B. abortus* may be present in the blood of equines and maintained for long periods of time without much change, or without any symptom of disease made manifest.

LABORATORY REQUIREMENTS IN THE DIAGNOSIS OF PULMONARY TUBERCULOSIS. *Wendell J. Stainsby, M.D.* George F. Geisinger Memorial Hospital, Danville, Pa.

The "direct smear" examination of sputum for tubercle bacilli is a valuable diagnostic procedure, as it takes little time to perform and gives immediate results in grossly infected specimens. A "concentration" examination such as that recommended by Petroff should be used when the "direct smear" is negative, and in the average laboratory will increase the positive results immediately obtained by from 10 to 15 per cent. Before a patient's sputum is considered negative, guinea pig inoculation or culture of the specimen should be carried out. In a series of 171 specimens obtained from patients suspected of pulmonary tuberculosis but which were negative by "concentration" examination, comparative tests were carried out simultaneously. Twenty-two of these were positive in both guinea pig and culture; 9 were positive by culture only and 4 were positive by guinea pig only. Petragnani's medium was used for the culture work. From these results it is apparent that guinea pig inoculations or cultures for tubercle bacilli are almost equally valuable for demonstrating tubercle bacilli in specimens containing few organisms. The examination by "direct smear" or "concentration" of an early morning gastric washing from patients, suspected of pulmonary tuberculosis but who cannot provide a suitable specimen, often demonstrates the presence of tubercle bacilli.

THE BACTERIOSTATIC ACTION OF SULFANILAMIDE UPON HEMOLYTIC STREPTOCOCCI OF HUMAN ORIGIN. *Erwin Neter*, Children's Hospital—Buffalo, N. Y., and University of Buffalo Medical School.

The bacteriostatic action of various concentrations of sulfanilamide (prontylin, repurified for injection) upon different strains of hemolytic streptococci of human origin, was tested in a culture medium containing phenol red broth base (Difco), 1% glucose and maltose respectively and 1% soluble starch. One loopfull of a 16- to 18-hour brain-heart-infusion broth culture of the respective organism was used for inoculation.

In the control broths without sulfanilamide, growth and acid formation occurred within 8–18 hours. In the presence of 0.8% sulfanilamide, growth and acid formation of certain strains of hemolytic streptococci were completely inhibited, even when the culture tubes were incubated for three weeks at 37°C. Smaller concentrations of sulfanilamide, ranging from 0.08%–0.000008% retarded the growth of sulfanilamide-susceptible strains of hemolytic streptococci, but did not prevent it. Hemolytic enterococci, and a few strains of non-fibrinolytic hemolytic streptococci of human origin, were not at all or only slightly inhibited by sulfanilamide in parallel experiments.

THE EFFECT OF THYROID ACTIVITY ON THE PRODUCTION OF AGGLUTININS FOR STREPTOCOCCUS PYOGENES. *Lysetta Marshak*, Bacteriological Laboratory, Bucknell University.

An attempt was made in this work to establish the relation between thyroid activity and the production of agglutinins for hemolytic streptococci. Eight rabbits of approximately the same weight were tested for the presence of natural agglutinins. Since no native agglutinins were found, these animals were used in the following experimental work. Three rabbits were thyroidectomized; two others were fed dessicated thyroid each week on the day preceding inoculation. The thyroid was bared in another rabbit which then acted as the operative control. Two others were used as regular controls. Immunity to the streptococci was established by administering graded doses weekly of killed organisms for four weeks and of viable organisms for three weeks thereafter. The rabbits were bled, and agglutination tests were run on their sera with extreme variability in results. Finally, an attempt was made to kill the rabbits by mass doses, but four of the animals did not succumb even to seventy billion virulent organisms and were, therefore, sacrificed. This last step served as a check on the agglutination results and also indicated, as did the agglutination tests run on the last four rabbits before they were killed, that agglutinin was not the immune substance responsible for the ability of the rabbits to resist such large doses of virulent streptococci. The conclusions, based on the agglutination tests and the order in which the rabbits died, were that thyroid upset, either hypo- or hyperthyroidism, has no specific effect on the immunity mechanism of the rabbit and causes, at best, a slight increase in the agglutination titre of those animals in which the thyroid balance has been upset.

A Study of the Lipids of Several Strains of Enteric Bacilli. *Clara I. Hinton, Leslie A. Sandholzer, and Walter R. Bloor*, Dept. of Bacteriology, University of Rochester, Rochester, N. Y.

A study has been made of the lipids of nine strains of enteric bacilli. Seven of these strains were members of the *Escherichia* genus, one was a typical *Eschcrichia-Aerobacter* Intermediate and one was a strain of *Shigella paradysenteriae* (Flexner). The organisms were grown on a fat-free agar medium under constant conditions. They were harvested, washed, frozen and dried *in vacuo* from the frozen state. The analyses for lipids were made on the whole dried organisms by Bloor's micromethods.

The total lipid of the dried organisms varied from 4.4 to 7.9 per cent. It consisted largely of phospholipid. The neutral fat represented 1 per cent or less of the total lipid. No sterols were found.

Phospholipid, present in approximately equal amounts in all of the organisms, represented about 4.5 per cent of the dry weight of the bacteria. Although similar to other phospholipids in fatty acid-, phosphorus- and nitrogen-content, the phospholipids from the various strains varied widely in their iodine numbers, indicating marked differences in the structure of the fatty acids.

The Amylase and Phosphatase Tests in Relation to the Commercial Pasteurization of Milk. *Harold W. Leahy*, Rochester Health Bureau Laboratories, Department of Bacteriology, University of Rochester School of Medicine and Dentistry Rochester, New York.

A modified Scharer Phosphatase Test and the author's Amylase Test were compared on 926 samples of milk collected at various stages of pasteurization from 137 vats in 90 pasteurizing plants. The modified Scharer's procedure compared favorably with that of Kay and Graham, but was more sensitive than the author's Amylase Test or the original method for detecting underheating or the admixture of raw milk. The modified method and the author's Amylase Test failed to detect the presence of milk pasteurized at 143°F for 15 minutes in 20.4 per cent and 37.9 per cent, respectively, of the vats examined.

Modified Scharer Test: 1 cc. of milk was added to 10 cc. of Kay and Graham's disodium phenylphosphate-sodium veronal solution, a drop of chloroform added, the mixture incubated at 37°C for 18 hours, and 0.2 cc. of a 0.4 per cent solution of 2,6-dibromoquinonechloromide in 95 per cent alcohol added. Properly pasteurized milk produced a slight greyish color, while the addition of 0.2 per cent of raw milk, or a slight underheating (1° to 2°F), or a shortening of the holding period (5 or 10 minutes), produced a blue color, the intensity of which depended upon the degree of underheating or the amount of raw milk present.

Physical Chemistry as Applied to Bacteriology. *M. W. Lisse,* Department of Agricultural and Biological Chemistry, The Pennsylvania State College, State College, Pa.

Some Characteristics of Strains of Rhizobium as Related to their Previous Symbiotude. *H. J. Webb,* Department of Agronomy, Cornell University, Ithaca, N. Y.

Types of Nodules Produced on Species of Legumes by Various Strains of Rhizobium. *J. K. Wilson,* Department of Agronomy, Cornell University, Ithaca, N. Y.

Multiple Virus Infection of Individual Host Cells. *Jerome T. Syverton and George Packer Berry,* School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

Concentrations of Zinc Influencing Biological Activities in Soil. *W. L. Lott,* Department of Agronomy, Cornell University, Ithaca, New York.

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